

**1871288CD1\_PRT\_17\_PF-0637-PCT**

252 aa

**TMEPAI**

287 aa

Transmembrane prostate androgen induced RNA, binds the WW domains of NEDD4 ubiquitin-protein ligase, expression is stimulated by TGFbeta, EGF, and other growth factors, several isoforms exist, altered expression is associated with numerous types of cancer

Match: Length=252, Identity: 99%, Similarity:99%, Query Overlap: 100%, Subject Overlap: 87%, E-value:1e-152, Score:520

Query: 1 MAELEFVQIIIIIVVVMMVMVVITCLLSHYKLSARSFISRHSQGRRREDALSSEGCLWPS 60  
+ ELEFVQIIIIIVVVMMVMVVITCLLSHYKLSARSFISRHSQGRRREDALSSEGCLWPS

Sbjct: 36 ITELEFVQIIIIIVVVMMVMVVITCLLSHYKLSARSFISRHSQGRRREDALSSEGCLWPS 95

Query: 61 ESTVSGNGIPEPQVYAPPRPTDRLAVPPFAQRERFHRFQPTYPYLQHEIDLDPPTISLSDG 120  
ESTVSGNGIPEPQVYAPPRPTDRLAVPPFAQRERFHRFQPTYPYLQHEIDLDPPTISLSDG

Sbjct: 96 ESTVSGNGIPEPQVYAPPRPTDRLAVPPFAQRERFHRFQPTYPYLQHEIDLDPPTISLSDG 155

Query: 121 EEPYYQGPCTLQLRDPEQQLELNRESVRAPPNRTIFDSDLMDSARLGGCPSSNSGIS 180  
EEPPPYQGPCTLQLRDPEQQLELNRESVRAPPNRTIFDSDLMDSARLGGCPSSNSGIS

Sbjct: 156 EEPYYQGPCTLQLRDPEQQLELNRESVRAPPNRTIFDSDLMDSARLGGCPSSNSGIS 215

Query: 181 ATCYGSGRMEGPPPTYSEVIGHYPGSSFQHQQQSSGPPSLLEGTRLHHTHIAPLESAAIW 240  
ATCYGSGRMEGPPPTYSEVIGHYPGSSFQHQQQSSGPPSLLEGTRLHHTHIAPLESAAIW

Sbjct: 216 ATCYGSGRMEGPPPTYSEVIGHYPGSSFQHQQQSSGPPSLLEGTRLHHTHIAPLESAAIW 275

Query: 241 SKEDKQKGHPL 252

SKEDKQKGHPL

Sbjct: 276 SKEDKQKGHPL 287

## Schematic Colors:

Very Strong	Strong	High	Moderate	Low	Weak
>95%	80-95%	45-80%	35-45%	25-35%	20-25%

**Human protein: Q8NER4 - PMEPA1 variant A protein. EMBL**



**Bioinformatic Harvester (c)**

Idea? Comment? Inconsistency?

**FORUM**

Length: **237 aa**, molecular weight: **26201 Da**, CRC64 checksum: **A44A274EAABFD930**

MMVMVVVITC	LLSHYKLSAR	SFISRHSQGR	RREDALSSSEG	CLWPSESTVS	GNGIPEPQVY	60
APPRPTDRLA	VPPFAQRERF	HRFQPTYPYL	QHEIDLPTTI	SLSDGEEPPP	YQGPCTLQLR	120
DPEQQLELNR	ESVRAPPNRT	IFDSSDLMDSA	RLGGPCPPSS	NSGISATCYG	SGGRMEGPPP	180
TYSEVIGHYP	GSSFQHQQSS	GPPSLLEGTR	LHHTHIAPLE	SAAIWSKEKD	KQKGHPL	237

//

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AY128643**



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GeneReport  
AY128643

**TMEPAI**

**transmembrane, prostate androgen induced RNA**  
[UniGene](#), [LocusLink](#), [OMIM](#), [GenAtlas](#), [GeneCard](#), [Ensembl](#), [MapView](#),  
[Genome Browser](#)

**Aliases**

- PMEPA1
- TRANSMEMBRANE PROSTATE ANDROGEN-INDUCED RNA

**Chromosomal Location**

Chromosome/Cytoband	20q13.31-q13.33
---------------------	-----------------

**Microarray Gene Expression Data**

Data available	Show Gene Expression Data
----------------	---------------------------

**SwissProt Information**

SwissProt Accession No.	<b>Q969W9</b> Transmembrane prostate androgen-induced protein (Homo sapiens)
-------------------------	--

Subcellular Location	type ib membrane protein (potential).
----------------------	---------------------------------------

Subunit	interacts with the ww domains of nedd4 (by similarity).
---------	---

<b>Tissue Specificity</b>	highest expression in prostate. also expressed in ovary.
<b>Miscellaneous</b>	alternative products: event=alternative splicing; named isoforms=2; name=1; isoid=q969w9-1; sequence=displayed; name=2; isoid=q969w9-2; sequence=vsp_006438; note=no experimental confirmation available;
<b>Induction</b>	by androgen.
<b>Similarity</b>	belongs to the tmepai family.
<b>SwissProt Copyright</b>	This SWISS-PROT entry is copyright. It is produced through a collaboration between the Swiss Institute of Bioinformatics and the EMBL outstation - the European Bioinformatics Institute. There are no restrictions on its use by non-profit institutions as long as its content is in no way modified and this statement is not removed. Usage by and for commercial entities requires a license agreement (See <a href="http://www.isb-sib.ch/announce/">http://www.isb-sib.ch/announce/</a> or send an email to <a href="mailto:license@isb-sib.ch">license@isb-sib.ch</a> ).

## Annotations

Gene Ontologies	<b>Ontology</b>	<b>Annotation</b>	<b>Evidence</b>	<b>Source</b>
	<b>Molecular Function</b>	Molecular function unknown	ND	GOA
	<b>Biological Process</b>	Androgen receptor signaling pathway	NAS	GOA
	<b>Cellular Component</b>	Integral to membrane	NAS	GOA

## UniGene & EST Expression Information

UniGene Cluster	Hs.83883 from Build No. 173 , Released on 2004-09-06		
Normalized expression distribution for tissue type Top ten [of 28] [Help]	Tissue	Normalized Expression (%)	Cluster Clones Tissue clones
Pancreas:	16.86	50:67592	
Soft_Tissue:	16.78	5:6792	
Prostate:	9.92	42:96523	
Colon:	5.82	21:82275	
Embryonic Stem cells:	4.73	1:4820	
Bone:	4.45	10:51252	
Pooled:	4.00	1:5703	
Eye:	3.18	18:128990	
Muscle:	3.01	12:90826	
Stomach:	2.86	9:71656	

SAGE (NCBI)

Go to Gene-to-tag Mapping at NCBI

## Upstream Genomic Sequence

TRASER	Upstream genomic sequence for transmembrane, prostate androgen induced RNA
--------	--

## Representative mRNA Sequences

UniGene	NM_020182	
	Accession	Description
	NM_020182	This variant (1) represents the longest transcript and encodes the longest isoform (a).
		This variant (4) differs in the 5' UTR and coding region compared to variant 1. The resulting isoform (c) is

LocusLink RefSeq

<u>NM_199171</u>	shorter compared to isoform a. Transcripts 3 and 4 both encode isoform c.
<u>NM_199169</u>	This variant (2) differs in the 5' UTR and coding region compared to variant 1. The resulting isoform (b) is shorter and has a distinct N-terminus compared to isoform a.
<u>NM_199170</u>	This variant (3) differs in the 5' UTR and coding region compared to variant 1. The resulting isoform (c) is shorter compared to isoform a. Transcripts 3 and 4 both encode isoform c.

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**NCBI**

	BLAST	Protein	Structure	PubMed	Taxonomy
	Genome	Nucleotide	3D-Domains	Books	Help

**Query: gi|22121999 PMEPA1 variant A protein [Homo sapiens]**  
**Matching gi: 40317620, 40317618**

[Best hits](#) [Common Tree](#) [Taxonomy Report](#) [3D Structures](#) [CDD Search](#)

**85 BLAST hits to 24 unique species Sort by BLAST score**

Archaea  Bacteria  Metazoa  Fungi  Plants  Viruses  Other Eukaryotes

Keep only  Cut-Off 80  Select  Reset

237 aa

SCORE	P	ACCESSION	GI	PROTEIN DESCRIPTION
Conserved Domain Database hits				
1282	27	AAH15918	45946553	TMEPAI protein [Homo sapiens]
1282	27	NP_954638	40317616	transmembrane prostate
1282	27	NP_064567	21361841	transmembrane prostate
1274	27	AAH80635	51593771	Transmembrane prostate
1096	27	CAB55862	5911816	dJ18J7.1 (androgen inc)
781	27	NP_852146	41281943	chromosome 18 open read
781	27	NP_004329	4757884	chromosome 18 open read
781	27	NP_001003	51093708	chromosome 18 open read
761	27	AAH66971	44890592	C18orf1 protein [Homo sapiens]
721	27	NP_852148	41281948	chromosome 18 open read
721	27	NP_001003	51093710	chromosome 18 open read
721	27	NP_852147	41281957	chromosome 18 open read
186	27	CAC32857	13160408	dJ1059L7.1.2 (androgen)
186	27	CAB88144	7619746	dJ1059L7.1.1 (androgen)
93	27	CAD97884	31873900	hypothetical protein [Homo sapiens]
92	27	AAQ98856	37693041	transducer of regulated
92	27	AAH23614	33874587	MECT1 protein [Homo sapiens]
92	27	T00388	7513044	hypothetical protein K1
92	27	AAH17075	31455200	MECT1 protein [Homo sapiens]
92	27	NP_056136	34732709	mucoepidermoid carcinoma
84	27	P54259	29429203	Atrophin-1 (Dentatorubro-pallidoluysian atrophy)

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ACTIVATE: SMART analysis

**No domains, repeats, motifs or features could be predicted with confidence**

These features and domains are not shown in the diagram, either because their scores are less significant than the required threshold, or because they overlap with some other source of annotation:

	name	begin	end	E-value	reason
Pfam:Drf_FH1		44	205	7.50e-01	threshold
IB		150	212	3.19e+03	threshold
THY		207	233	2.18e+03	threshold

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### UniGene Cluster Hs .83883 *Homo sapiens*

**Transmembrane, prostate androgen induced RNA (TMEPAI)**

[Links](#)

### SELECTED PROTEIN SIMILARITIES

organism, protein and percent identity and length of aligned region

*H.sapiens*: [ref:NP\\_004329.1](#) - chromosome 18 open reading frame 1; 67.60 % / 249 aa  
clone 22 [*Homo sapiens*]  
(see [ProtEST](#))

*M.musculus*: [ref:NP\\_075371.1](#) - Nedd4 WW binding# protein 4; Nedd4 88.10 % / 252 aa  
WW-binding protein 4 [*Mus musculus*]  
(see [ProtEST](#))

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**e! Ensembl Human Gene View**

The Wellcome Trust Sanger Institute EMBL

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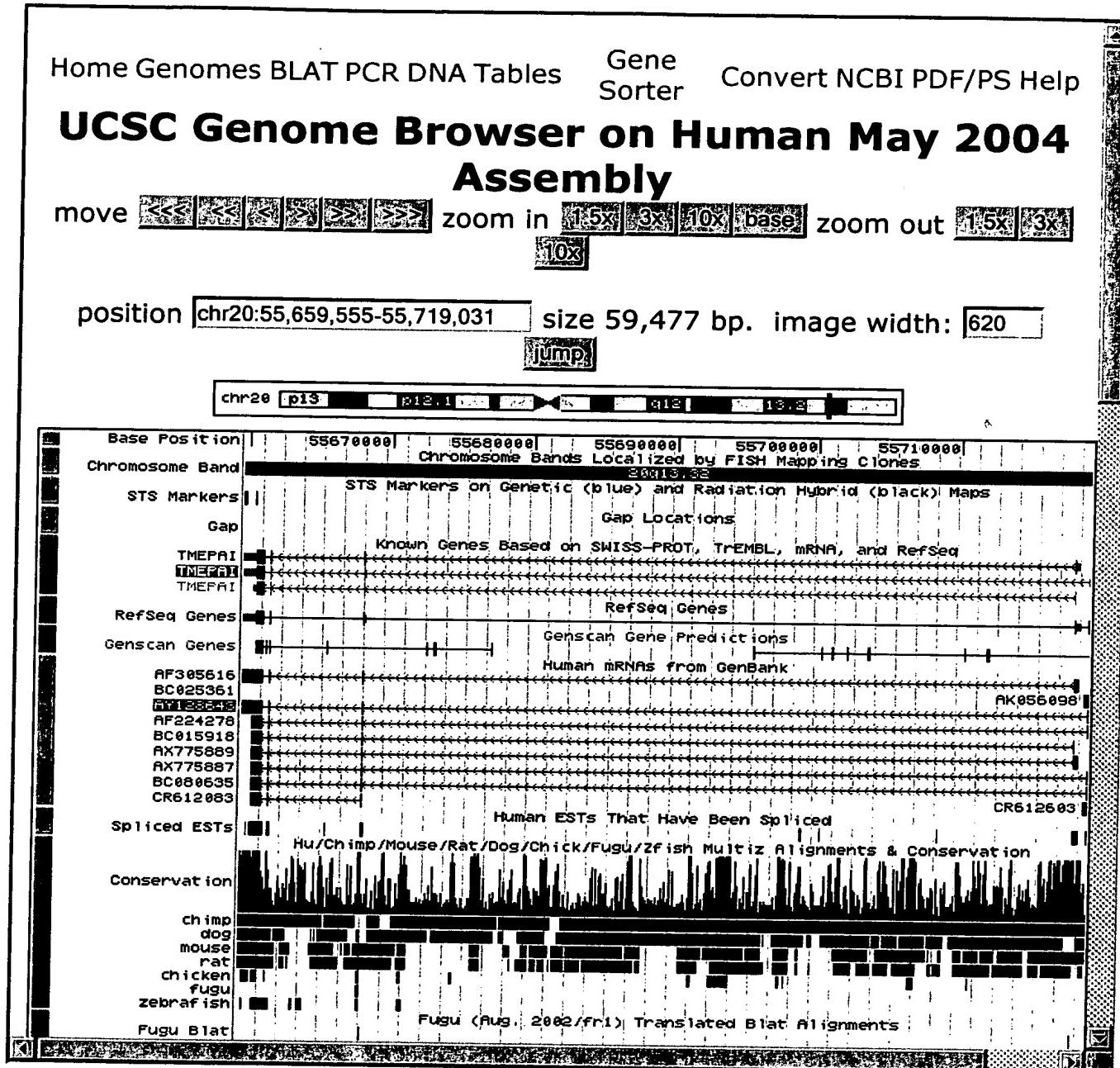
Find    [e.g. ENSG00000139618, BRCA2]

## Ensembl Gene Report

Gene	TMEPAI (HUGO ID)
Ensembl Gene ID	ENSG00000124225
Genomic Location	<a href="#">View gene in genomic location: 56908875 - 56971962 bp (56.9 Mb) on chromosome</a> <a href="#">This gene is located in sequence: AL121913.4.1.150224</a>
Description	Transmembrane prostate androgen-induced protein (Solid tumor- associated 1 protein SWISSPROT (Q969W9))
Prediction Method	Genes were annotated by the Ensembl automatic analysis pipeline using either a Gene from a human/vertebrate protein, a set of aligned human cDNAs followed by Genome prediction or from Genscan exons supported by protein, cDNA and EST evidence. Genes are further combined with available aligned cDNAs to annotate UTRs.
Sequence Markup	<a href="#">View genomic sequence for this gene with exons highlighted</a>
Export Data	<a href="#">Export gene data in EMBL, GenBank or FASTA</a>
SNP information	<a href="#">View information about variations on this gene.</a>
	1: TMEPAI (ENST00000265626) <a href="#">[Transcript information]</a> <a href="#">[Exon information]</a> <a href="#">[Protein information]</a> 2: TMEPAI (ENST00000341744) <a href="#">[Transcript information]</a> <a href="#">[Exon information]</a> <a href="#">[Protein information]</a> 3: TMED11 (ENST00000247015) <a href="#">[Transcript information]</a> <a href="#">[Exon information]</a> <a href="#">[Protein information]</a>

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9/23

60.9 %: nuclear  
13.0 %: mitochondrial

13.0 %: plasma membrane  
8.7 %: cytoplasmic  
4.3 %: vesicles of s

45

secretory system

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Search across databases

none  **PubMed:** biomedical literature citations and abstracts

none  **PubMed Central:** free, full text journal articles

none  **Books:** online books

none  **OMIM:** online Mendelian Inheritance in Man

none  **Site Search:** NCBI web and FTP sites

1  **Nucleotide:** sequence database (GenBank)

none  **Protein:** sequence database

none  **Genome:** whole genome sequences

none  **Structure:** three-dimensional macromolecular structures

none  **Taxonomy:** organisms in GenBank

none  **SNP:** single nucleotide polymorphism

1  **Gene:** gene-centered information

none  **HomoloGene:** eukaryotic homology groups

none  **PubChem Compound:** small molecule chemical structures

none  **PubChem Substance:** chemical substances screened for bioactivity

none  **UniGene:** gene-oriented clusters of transcript sequences

none  **CDD:** conserved protein domain database

none  **3D Domains:** domains from Entrez Structure

3  **UniSTS:** markers and mapping data

none  **PopSet:** population study data sets

22  **GEO Profiles:** expression and molecular abundance profiles

none  **GEO DataSets:** experimental sets of GEO data

none  **Cancer Chromosomes:** cytogenetic databases

none  **PubChem BioAssay:** bioactivity screens of chemical substances

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EMBL/Genbank->Unigene mapping provided by RZPD <http://www.rzpd.de>

 Deutsches  
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für Genomforschung  
GmbH

### Gene Information and Ordering of RZPD Products for **TMEPAI (Transmembrane, prostate andro- induced RNA)**

NCBI Unigene Cluster [Hs.83883](#) Homo sapiens Version : #175

Title Transmembrane, prostate androgen induced RNA

Gene **TMEPAI**

Locus Link [56937](#)

Chromosome Location 20:q13.31-q13.33

RefSeq [NM\\_020182](#)  
[NM\\_199169](#)  
[NM\\_199170](#)  
[NM\\_199171](#)

Further Gene Links [GeneCards](#) [GeneNest](#) [S.O.U.R.C.E.](#) [SYSTEMS](#) [Swiss-Prot](#)

Cross Reference Unigene - Ensembl (Hs#24\_34e)

Ensembl Gene ID [ENSG00000124225](#)

Complex Gene Annotation [GenomeMatrix](#) [OMIM ID:606564](#)

Reference IDs to Unigene Gene Symbol : TMEPAI Locus Link : 56937 RefSeq : NM\_020182,NM\_199169,NM\_199170,NM\_199171

### EBI-Hinxton-"Uniprot-Swissprot-TrEMBL" database

#### **General information**

[www.uniprot.org](http://www.uniprot.org) [Q8NER4](#) | [XML](#)

Entry name **Q8NER4**

Accession number **Q8NER4**

Created TrEMBLrel. 22, 1-OCT-2002

Sequence update TrEMBLrel. 22, 1-OCT-2002

Annotation update TrEMBLrel. 24, 1-JUN-2003

#### **Description and origin of the Protein**

Description PMEPA1 variant A protein.  
Organism source Homo sapiens (Human).  
Taxonomy Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

NCBI TaxID 9606

**References**

- [1] Brunschwig,E.B., Wilson,K., Mack,D., Dawson,D., Lawrence,E., Willson,J.K., Lu,S., Nosrati,A., Rerko,R.M., Swinler,S., Beard,L., Lutterbaugh,J.D., Willis,J., Platzer,P., Markowitz,S., **PMEPA1, a transforming growth factor-beta-induced marker of terminal colonocyte differentiation whose expression is maintained in primary and metastatic colon cancer.**  
(2003) *Cancer Res.* **63**:1568-1575  
Position SEQUENCE FROM N.A.  
Medline [22557253](#)  
PubMed [12670906](#)

**Database cross-references**

EMBL [AY128643](#); [AAM89277.1](#); -.

GO [GO:0016021](#); C:integral to membrane; NAS.  
[GO:0030521](#); P:androgen receptor signaling pathway; NAS.

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GoTo: SOSUI analysis

This amino acid sequence is of a MEMBRANE PROTEIN  
which have 1 transmembrane helix.

No.	N terminal	transmembrane region	C terminal	type	length
1	2	MVMVVVITCLLSHYKLSARFIS	24	PRIMARY	23

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Your Input:  
ENSP00000326920 annotation not available (267 aa)

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Views: Neighborhood Fusion Cooccurrence Coexpression Experiments Databases

note: you bypassed the normal STRING input page.  
For your information, the sequence you provided was not found as such.  
However, a similarity search indicated a close match to the protein 'ENSP00000326920'

**Info & Parameters...**

Network Display - Nodes (orthologous groups) are either coloured directly linked to the input - as in the table) or white (nodes are not linked to the input). Edges (putative links between orthologous groups) consist of up to three lines: a red line indicates the presence of fusion evidence for the link; green - neighbourhood evidence; blue - physical cooccurrence evidence. A bold line indicates particularly strong evidence in each case. Hover or click to reveal more information about the nodes.

Active Prediction Methods:

Neighborhood  Gene Fusion  Co-occurrence  
 Co-expression  Experiments  Databases  Textmining

required confidence (score):  medium confidence (0.400)   
or custom value:  interactors shown:  no more than 10 interactors

edge scaling factor  80%   
network depth  1

# PMEPA1, an Androgen-regulated NEDD4-binding Protein, Exhibits Cell Growth Inhibitory Function and Decreased Expression during Prostate Cancer Progression<sup>1</sup>

Linda L. Xu, Yinghui Shi, Gyorgy Petrovics, Chen Sun, Mazen Makarem, Wei Zhang, Isabell A. Sesterhenn, David G. McLeod, Leon Sun, Judd W. Moul, and Shiv Srivastava<sup>2</sup>

*Center for Prostate Disease Research, Department of Surgery, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799 [L. L. X., Y. S., G. P., C. S., M. M., W. Z., D. G. M., J. W. M., S. S. J.], and Urology Service, Walter Reed Army Medical Center [D. G. M., J. W. M.] and Department of Genitourinary Pathology, Armed Forces Institute of Pathology [I. A. S. J.], Washington, DC 20307*

## Abstract

*PMEPA1* was originally identified as a highly androgen-induced gene by serial analysis of gene expression in androgen-treated LNCaP prostate cancer (CaP) cells. *PMEPA1* expression is prostate abundant and restricted to prostatic epithelial cells. *PMEPA1*-encoded protein shows high sequence homology to a mouse *N4wbp4*-encoded protein that binds to Nedd4 protein, an E3 ubiquitin-protein ligase involved in ubiquitin-dependent, proteasome-mediated protein degradation. Studies from our and other laboratories have suggested the role of *PMEPA1* in cell growth regulation as noted by androgen induction of *PMEPA1* expression, elevated *PMEPA1* expression in nontumorigenic revertants of tumor cell lines after chromosome 8p transfer, and *PMEPA1* expression alterations (up- or down-regulation) in human tumors. Here, we demonstrate that *PMEPA1* protein through its PY motifs interacts with WW domains of the human NEDD4 protein. Exogenous expression of *PMEPA1*, in widely used CaP cell lines DU145, PC3, LNCaP, and LNCaP sublines (C4, C4-2, and C4-2B), conferred cell growth inhibition, and at least one of the PY motifs of *PMEPA1* may be involved in its cell growth inhibitory functions. Quantitative expression analysis of *PMEPA1* in paired normal and tumor cells of 62 patients with primary CaP revealed tumor cells associated decreased expression in 40 of 62 patients that were significantly associated with higher pathologic stage and serum prostate-specific antigen. Taken together, *PMEPA1* negatively regulates growth of androgen responsive or refractory CaP cells, and these functions may be mediated through the interaction of *PMEPA1* with the NEDD4 protein involved in the ubiquitin-proteasome pathway. Loss or reduced *PMEPA1* expression in CaP further suggests for its role in prostate tumorigenesis.

## Introduction

Biological effects of androgens on target cells, e.g., prostatic epithelial cell proliferation and differentiation, as well as androgen ablation-mediated cell death, involve AR-mediated<sup>3</sup> cell signaling (1). Systematic and comprehensive analysis of the ARGs should provide the biological reporters for androgen signaling in CaP. Our efforts to analyze ARGs by serial analysis of gene expression led to the dis-

covery of *PMEPA1* (2, 3). *PMEPA1* expression was regulated by androgen in a dose- and time-dependent manner, and *PMEPA1* was highly expressed in the prostate in comparison with other organs.

*PMEPA1* encodes a protein of 252 amino acids with a type Ib trans-membrane domain. *PMEPA1* protein sequence homology search showed 83% identity to a recently reported mouse *N4wbp4* protein, which was defined as one of the several proteins that bound to WW domain of the *Nedd4*-encoded protein (4). *Nedd4*, originally identified as a developmentally regulated gene in mice, now belongs to a family of ubiquitin-protein ligases characterized by two to four WW domains, a COOH-terminal homologous to E6-AP COOH terminus domain and an NH<sub>2</sub>-terminal C2 domain (5–7). Additional studies implicated the roles of *Nedd4* in diverse cellular functions through the ubiquitin-dependent, proteasome-mediated protein degradation (8–12). The WW domain of the *Nedd4* protein comprises of a module with two highly conserved tryptophan residues, which bind to target proteins that contain a PY motif, e.g., PPXY (4, 13–17). The presence of two PY motifs in the predicted protein sequence of *PMEPA1* and its similarity to the mouse *Nedd4*-binding protein, *N4wbp4*, suggest that *PMEPA1* is a potential binding partner of the NEDD4, the human homologue of the *Nedd4*.

Studies of *PMEPA1* (2, 3, 18, 19) have suggested for its role in cell growth regulation as noted by the androgen induction of *PMEPA1* expression, elevated *PMEPA1* expression in nontumorigenic revertants of tumor cell lines after chromosome 8p transfer, and the *PMEPA1* expression alterations (up- or down-regulation) in human tumors. Furthermore, the induction of *PMEPA1* expression in nontumorigenic derivatives of multiple cancer cell lines, resulting from the introduction of chromosome 8p, suggested that *PMEPA1* might be the downstream target of the critical cell growth regulatory genes on chromosome 8, the most frequently altered chromosomes in CaP.

The androgen-regulated nature of *PMEPA1*, the potential of *PMEPA1* protein as a NEDD4-binding partner and suggested cell growth regulatory functions of *PMEPA1*, have now provided the impetus to study biochemical and cell biological functions of *PMEPA1* and its CaP-associated alterations.

## Materials and Methods

**Plasmids.** Mammalian expression vectors encoding *PMEPA1*-V5 and *PMEPA1*-GFP fusion proteins were generated by PCR amplification of the *PMEPA1* open reading frame. For *PMEPA1*-V5-pcDNA3.1 vector, the primers 5'-GCTGCTGGAGAACTGAAGCG3' and 5'-GTGTCCTTCTGTTATC-CTTC3' were used. For *PMEPA1*-GFP-pEGFP vector, the primers used were 5'-aaggctGCTGCTGGAGAACTGAAGG CG 3' and 5'-gaattcGGTGCCTT-TCTGTTATC3'. The V5 tag or GFP protein was fused at the COOH terminus of the *PMEPA1* protein. The PCR product for generating *PMEPA1*-V5 was inserted into pcDNA3.1-V5-His expression vector (Invitrogen, Carlsbad, CA). The PCR product for generating *PMEPA1*-GFP was

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<sup>1</sup> Supported by a grant from the Center for Prostate Disease Research, a program of the Henry M. Jackson Foundation for the Advancement of Military Medicine (Rockville, MD), funded by the United States Army Medical Research and Materiel Command.

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<sup>3</sup> The abbreviations used are: AR, androgen receptor; ARG, androgen-regulated gene; GFP, green fluorescence protein; CaP, prostate cancer; TSG, tumor suppressor gene; cT, cycle threshold; EGFP, enhanced green fluorescence protein; GST, glutathione S-transferase; TGN, trans-Golgi network; GAPDH, glyceraldehyde phosphate dehydrogenase; LCM, laser-capture microdissection; QRT-PCR, quantitative reverse transcription-PCR; wt, wild type; PSA, prostate-specific antigen.

digested by *Hind*III and *Eco*RI and cloned into the same sites of pEGFP vector (Clontech, Palo Alto, CA). PMEPA1-PY motif mutants, in which the tyrosine residue (Y) was replaced with an alanine residue (A), were created by using QuikChange Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) and using the *PMEPA1-V5*-pcDNA3.1 vector as a template. The plasmids of *PMEPA1-PY* motif mutants are as follows: (a) *PMEPA1-PY1m-V5*-pcDNA3.1, with the first PY motif mutation (Y126A); (b) *PMEPA1-PY2m-V5*-pcDNA3.1, with the second PY motif mutation (Y197A); and (c) *PMEPA1-PY1m/PY2m-V5*-pcDNA3.1, with both the PY motif mutations (Y126A and Y197A). The sequences of all of the inserts in expression vectors were verified by DNA sequencing.

A bacterial expression plasmid of human *NEDD4* gene (*pNEDD4WW-GST-pGEX-2TK*) encoding all four WW-domains (accession no. XM\_046129) fused to GST (GST-WW fusion protein) was generated by PCR amplification of the coding region of the four WW-domains using the primers 5'GCAG-GATCCCAACCAGATGCTGCTTCG3' and 5'GCAGAATTCTTTGTATCCCTGGAGTA3'. Normal prostate tissue-derived cDNA was used as a PCR template, and the amplified fragment was cloned into the *Bam*HI/*Eco*RI sites of pGEX-2TK (Amersham Biotech, Piscataway, NJ). A mammalian expression vector (*NEDD4-GFP-pEGFP*) encoding *NEDD4-GFP* fusion protein was generated using the primers 5'GCAAAGCTTGTCCGG TTTGCTG-GAACG3' and 5'GCAGAATTCCCTTTGTCTTATTGGTGAC3' to generate the *NEDD4* gene fragment by PCR.

**PMEPA1 and NEDD4 Protein-binding Assays.** The *in vitro* binding of PMEPA1 and NEDD4 was assessed by GST pull-down assays. GST-WW fusion protein was prepared and purified with glutathione-Sepharose beads per Amersham Biotech instructions. [<sup>35</sup>S]methionine-labeled proteins representing PMEPA1 and its mutants were generated by *in vitro* transcription/translation (TNT T7 quick coupled transcription/translation system; Promega, Madison, WI). Briefly, the *PMEPA1-V5*-pcDNA3.1 or three mutants (2 µg) were incubated in 40 µl of reticulocyte lysate with 40 µCi of [<sup>35</sup>S]methionine for 1.5 h at 30°C. [<sup>35</sup>S]methionine incorporation into protein was measured, and samples were equalized on the basis of cpm. The GST-WW fusion protein bound to glutathione-Sepharose beads (5 µg) was incubated with the [<sup>35</sup>S]methionine-labeled lysates (12 µl) in 0.4 ml of PBS (pH 7.4), 1 mM DTT, and protease inhibitors. The negative control for each [<sup>35</sup>S]methionine-labeled lysate represented a reaction mixture with equivalent amounts of the lysate incubated with glutathione-Sepharose beads without GST-WW fusion protein. After 16 h of incubation at 4°C, the beads were washed six times with PBS, resuspended in SDS-PAGE sample buffer, and run on 12% SDS-PAGE gel under a reducing condition. The gels were dried and autoradiographed.

The interaction of PMEPA1 and NEDD4 proteins in cells was evaluated by a coimmunoprecipitation assay. 293 cells (human embryonal kidney cells) were cotransfected with *NEDD4-GFP-pEGFP* vector and one of the *PMEPA1-V5* expression vectors encoding either wt PMEPA1-V5 or the PY mutants of *PMEPA1*. Thirty-six h later, the cells were collected and lysed, and the lysates were immunoprecipitated with anti-GFP antibody (Clontech) following the manufacturer's protocol. The immunoprecipitated proteins were subjected to immunoblotting with an anti-V5 tag antibody (Invitrogen).

**Immunofluorescence Assays.** These experiments were performed following the procedure described by Harvey *et al.* (17). Briefly, stable transfectants of LNCaP cells harboring *PMEPA1-GFP-pEGFP* (LNCaP-*PMEPA1-GFP* transfectant) were grown on coverslips for 2 days, fixed in 2% paraformaldehyde for 15 min, and permeabilized in 0.2% Triton X-100 for 2 min. Fixed and permeabilized cells were incubated with anti-GM130 (recognizes a *cis*-Golgi matrix protein) or anti-TGN38 (recognizes a protein localizing to TGN) monoclonal antibodies (BD Transduction Laboratory, San Diego, CA) at 6.25 µg/ml for 30 min at room temperature. Cells were then washed to remove excess or nonspecifically bound primary antibody followed by incubation with tetramethylrhodamine isothiocyanate-conjugated antimouse antibody (Sigma, St. Louis, MO) at 1:100 dilution for 30 min at room temperature. The sections were mounted with fluoromount (Southern Associates, Birmingham, AL), and the images were processed with a Leica fluorescence microscope and Open-Lab software (Improvision, Lexington, MA).

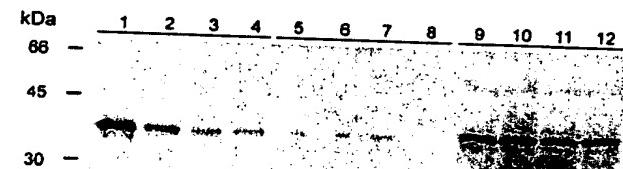
**Colony Formation Assays and Cell Proliferation Analysis.** Prostate cell lines LNCaP, PC3, and DU145 were purchased from American Type Culture Collection (Rockville, MD) and grown in the cell culture media as described by the supplier. The LNCaP sublines C4, C4-2, and C4-2B (20–22) were purchased from Urocor (Oklahoma, OK) and cultured in T medium (5% fetal

bovine serum, 80% DMEM, 20% F12, 5 µg/ml insulin, 13.65 pg/ml Triiodothyronine, 5 µg/ml apo-transferrin, 0.244 µg/ml biotin, and 25 µg/ml adenine). Three µg of plasmids (*PMEPA1-V5*-pcDNA3.1 or vector without *PMEPA1* insert) were transfected into the 50–70% confluent cells in triplicate in 60-mm Petri dishes with Lipofectamine (Invitrogen). TSG p53 (wt) and mt p53 (R175H and G245D) were also used in parallel as controls. Approximately 36 h later, selection with G418 at 800 µg/ml (DU145 and PC3) or 400 µg/ml (LNCaP and its sublines) was initiated. Cells were maintained with G418-containing medium that was changed every 3–4 days. After 2–4 weeks of selection, the cells were rinsed with 1× PBS, fixed with 2% formaldehyde in 1× PBS for 15 min, stained with 0.5% crystal violet in 1× PBS for 15 min,

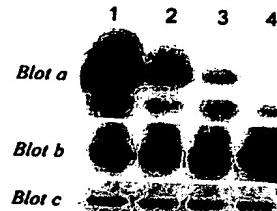
#### A

Human PMEPA1: 1	MAELEFVQIVVVVVVMMVVMITCLLSHYKLSARSFISRRSQRREDDALSSSEGLMP
	+ ELEPFVQIVVVVVVMMVVMITCLLSHYKLSARSFISRRSQRREDDALSSSEGLMP
Mouse N4wbp4: 18	ITELEPVQIVVVVVVMMVVMITCLLSHYKLSARSFISRRSQRREDDALSSSEGLMP
Human PMEPA1: 61	ESTVSGNGIPEPQVYAPPRPTDRLAVPPFQHFRHFRFQPTPYLYKEIILPPTISLSDG
Mouse N4wbp4: 78	ESTVSG+PEPQVYAPPRPTDRLAVPPFQHFRHFRFQPTPYLYKEIILPPTISLSDG
Human PMEPA1: 121	EEPFPPYQGPCTLQLRDPEQQLLNRSVRAFPHRTIFDQCLMDSARLGGPCPPSSNSG
Mouse N4wbp4: 134	EEPFPPYQGPCTLQLRDPEQQLLNRSVRAFPHRTIFDQCLIDSTNLGGPCPPSSNSG
Human PMEPA1: 181	ATCYGSGRNEGPPTYSEVIGHYPGSGSPQHQSSGPPSLLCETRHLHHNTHIAPILEGAA
Mouse N4wbp4: 194	ATCYSGCRNEGPPTYSEVIGHYPGSGSPQHQSSGPPSLLCETRHLHHNTHIAPILEGAA
Human PMEPA1: 241	SKEKDQKQKGHPL 252
Mouse N4wbp4: 249	NKEEKQKQKGHPL 260

#### B



#### C



**Fig. 1.** Homology of human PMEPA1 and mouse N4wbp4 protein sequences and the interaction of PY motifs of PMEPA1 with the WW domains of NEDD4. In A, PMEPA1 and N4wbp4 (GenBank accession no. AA008976) exhibit 83% sequence identity (analyzed through the National Center for Biotechnology Information Web site).<sup>4</sup> The + denotes conservative substitution. The PY motifs are underlined. B, *in vitro* transcription/translation products ([<sup>35</sup>S]Methionine-labeled lysates) derived from expression plasmids. PMEPA1-V5-pcDNA3.1 (Lanes 1 and 5), PMEPA1-PY1m-pcDNA3.1 (Lanes 2 and 6), PMEPA1-PY2m-pcDNA3.1 (Lanes 3 and 7), and PMEPA1-PY1m/PY2m-pcDNA3.1 (Lanes 4 and 8) were incubated with GST-NEDD4-WW-Sepharose beads (Lanes 1–4) or control GST beads (Lanes 5–8), and [<sup>35</sup>S]Methionine-labeled proteins bound to GST-NEDD4-WW-Sepharose beads were solubilized in sample buffer and resolved by SDS-PAGE gel. Equal amounts of [<sup>35</sup>S]Methionine lysates corresponding to samples in Lanes 1–4 were run on SDS-PAGE gel without GST pull-down (Lanes 9–12). In C, 293 cells were cotransfected with expression vectors encoding NEDD4-GFP and one of the following fusion proteins: PMEPA1-V5 (Lane 1), PMEPA1-PY1m-V5 (Lane 2), PMEPA1-PY2m-V5 (Lane 3), or PMEPA1-PY1m/PY2m-V5 (Lane 4). The cell lysates from each group were immunoprecipitated with anti-GFP antibody and then subjected to immunoblotting (Blot a). Cell lysates from each group without immunoprecipitation were also processed for immunoblotting (Blots b and c) to serve as control. Blots a and b were detected by anti-V5 antibody, and Blot c was detected by anti-GFP antibody.

<sup>4</sup> Internet address: [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).

and rinsed one to two times with distilled H<sub>2</sub>O. Colonies visible in each dish without magnification were counted by Open-Lab software. To assess the effects of PY motif mutations on the colony-forming ability of *PMEPA1*, LNCaP and PC3 cells were also transfected with *PMEPA1* mutants: *PMEPA1-PY1m*-pcDNA3.1, *PMEPA1-PY2m*-pcDNA3.1, or *PMEPA1-PY1m/PY2m*-pcDNA3.1. *PMEPA1-V5*-pcDNA3.1 and expression vector without insert served as positive and negative controls, respectively, for the *PMEPA1* mutants. Two independent colony-forming assays were performed as above.

To further evaluate the growth inhibitory effects of *PMEPA1* on CaP cells, a stable *PMEPA1*-GFP-Tet LNCaP transfectant was generated. Expression of *PMEPA1*-GFP fusion protein in these cells was negatively regulated by tetracycline in the medium (Clontech). For cell proliferation assays, 3000 1 µg/ml tetracycline in the medium. CellTiter 96 Aqueous One Solution kit (Promega) was used to measure the cell proliferation according to the manufacturer's instructions.

**Prostate Tissue Specimens, LCM, and QRT-PCR Assay.** Matched CaP and normal tissues were derived from radical prostatectomy specimens from 62 CaP patients treated at Walter Reed Army Medical Center (under an Institutional Review Board-approved protocol). The procedures for collecting specimens were described previously (23). Ten-µm frozen sections were prepared and archived at -70°C. Histologically normal prostate epithelial and prostate tumor cells from each patient were harvested by a pathologist (W. Z.) using LCM equipment according to the protocol provided by the manufacturer (Arcturus Engineering, Mountain View, CA). Total RNA was prepared from the harvested normal and tumor prostate epithelial cells as described previously (23) and quantified with Fluorometer (Bio-Rad, Hercules, CA). QRT-PCR was conducted using 0.1 ng of total RNA from paired normal and tumor cells. *PMEPA1* PCR primers were carefully designed that only amplify *PMEPA1* but not *STAC1*, an alternatively spliced form of *PMEPA1* (19). The PCR primers were 5'CATGATCCCCGAGCTGCT3' and 5'TGATCTGAA-CAAATCCAGCTCC3', and the FAM-labeled probe was 5'AGGCGGA-CAGTCTCTGCGAAC3'. GAPDH gene expression was detected as the internal control (PE Applied Biosystems, Foster, CA). Paired triplicate samples (one lacking reverse transcriptase and duplicate with reverse transcriptase) were amplified in 50-µl volumes containing the manufacturer's recommended universal reagent, proper primers, and probe of *PMEPA1* or *GAPDH* using 7700 sequence detection system (PE Applied Biosystems). Results were plotted as average cT values for each duplicate sample minus the average duplicate cT values for *GAPDH*. Differences between matched tumor (T) and normal (N) samples were calculated using  $2^{\exp(cT_{\text{tumor}} - cT_{\text{normal}})}$  and expressed as fold changes in expression. The expression status of *PMEPA1* was further categorized as overexpression in tumor tissue (T > N), defined as 1+ (1.5-3-fold), 2+ (3.1-10-fold), 3+ (10.1-20-fold), and 4+ (>20-fold) increased expression as compared with matched normal tissue; reduced expression in tumor tissue (T < N), defined as 1- (1.5-3-fold), 2- (3.1-10-fold), 3- (10.1-20-fold), and 4- (>20-fold) decreased expression as compared with matched normal tissue; and no change (T = N) refers to the difference of

*PMEPA1* expression between T and N as <1.5 folds (0). No detectable *PMEPA1* expression in one of the specimens of tumor/normal pairs was scored as 4+ for increased or 4- for decreased expression.

**Statistical Analysis.** Statistical analysis was performed with the SPSS software package. The association between *PMEPA1* expression and clinicopathological features was analyzed using  $\chi^2$  and t tests.  $P < 0.05$  was considered as statistically significant.

## Results and Discussions

**PMEPA1-PY Motifs Interact with the WW Domains of NEDD4.** The WW domains of NEDD4 protein facilitate its binding to the target proteins via interaction with the PY motifs of NEDD4-binding proteins (4, 13-17). Predicted *PMEPA1* protein sequence comprises of two PY motifs, PY1 (PPPY) and PY2 (PPTY). PY1 is in the central region of the *PMEPA1* protein, and PY2 is close to the COOH terminus of the *PMEPA1* protein (Fig. 1A). *PMEPA1* shares 83% sequence identity with the protein encoded by *N4wbp4*, a gene expressed in mouse embryo (Ref. 4; Fig. 1A). In vitro translated [<sup>35</sup>S]Methionine-labeled *PMEPA1-V5* fusion protein, with the two intact PY motifs, showed binding to the GST-WW fusion protein (Fig. 1B, Lane 1). *PMEPA1* with PY1 or PY2 mutations revealed significantly decreased binding to WW domains (Fig. 1B, Lanes 2 and 3). Furthermore, *PMEPA1-V5* and NEDD4-GFP fusion proteins expressed in 293 cells showed strong association (Fig. 1C, Lane 1) and the mutant *PMEPA1-V5* proteins having single mutation of PY1 or PY2 motif or double mutations of both PY1 and PY2 motifs exhibited significantly reduced binding to NEDD4 (Fig. 1C, Lanes 2-4). Thus, both *in vitro* and cell culture data support the prediction that *PMEPA1* interacts with NEDD4, and these interactions are dependent on the binding of both PY motifs of *PMEPA1* to WW domains. However, the PY2 motif mutation appeared to have a greater effect on binding of *PMEPA1* to NEDD4 WW domain.

The high protein sequence identity of *PMEPA1* with *N4wbp4* suggests that *PMEPA1* is the human homologue of *N4wbp4*. On the basis of the suggested role of Nedd4 in mouse development (4-12), the homology of *PMEPA1* to a Nedd4-binding protein, experimental documentation of binding of *PMEPA1* to NEDD4, and the androgen regulation and prostate abundance of *PMEPA1* warrant evaluations of the roles of *PMEPA1* in prostate development.

**PMEPA1 Is a Golgi-associated Protein.** To gain additional insights into *PMEPA1* cellular functions, its subcellular localization was determined. *PMEPA1*-GFP fusion protein showed perinuclear localization with a Golgi-like appearance (Fig. 2A). We performed the immunofluorescence assay to test the hypothesis that *PMEPA1* local-

### PMEPA1/GFP Transfectants

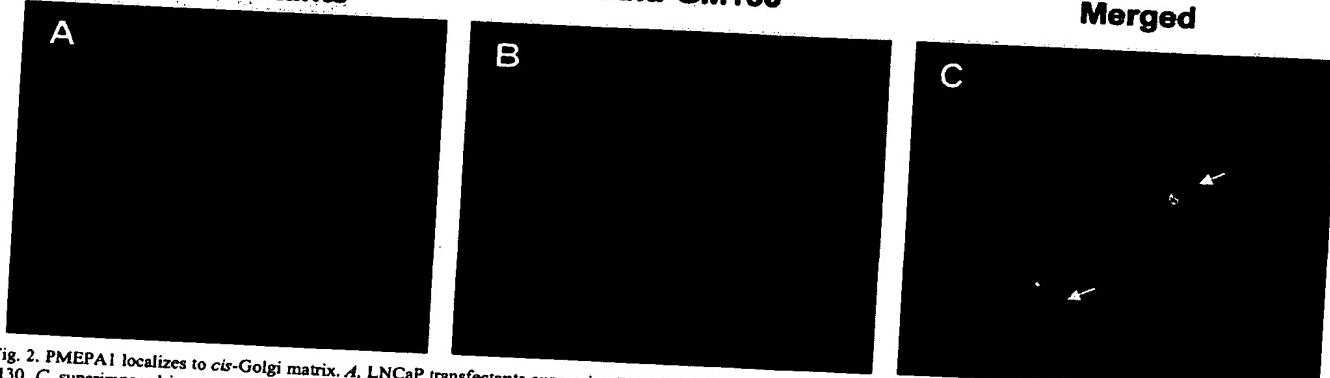
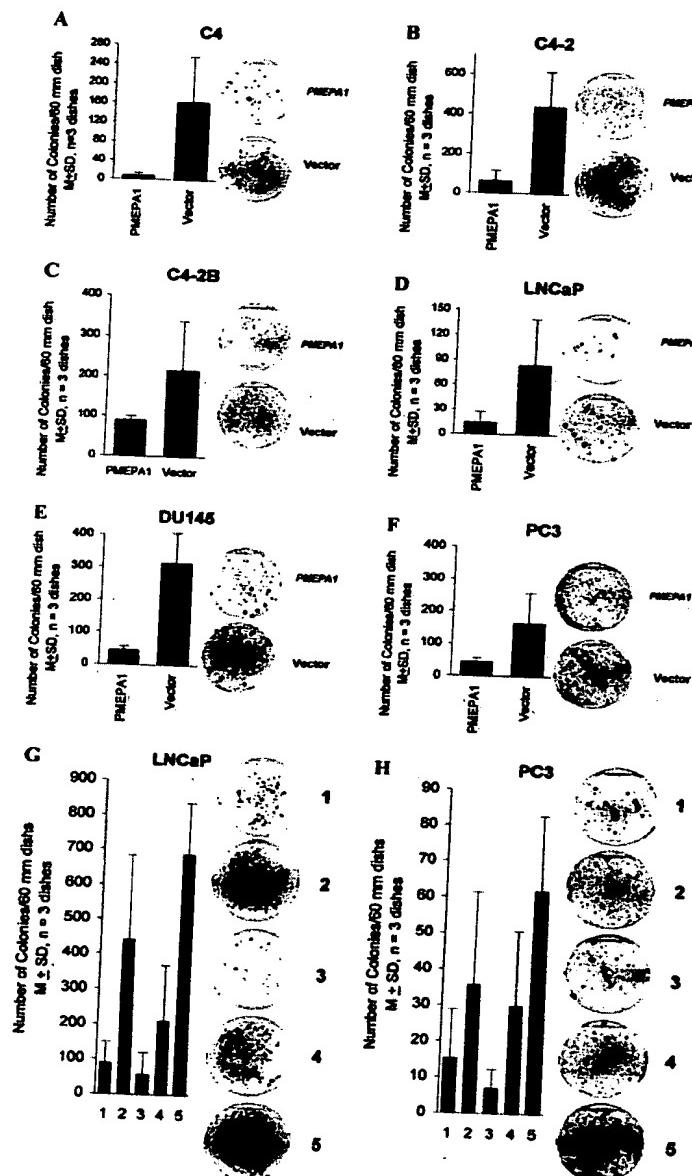
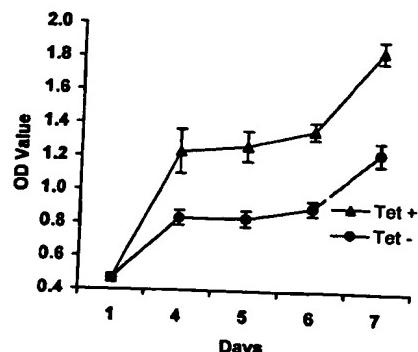


Fig. 2. *PMEPA1* localizes to *cis*-Golgi matrix. A, LNCaP transfectants expressing *PMEPA1*-GFP fusion protein. In B, LNCaP transfectants stained with anti-Golgi structure protein GM130. C, superimposed images of B and C. Arrows, the colocalization of *PMEPA1*-GFP fusion protein and GM130. Magnification:  $\times 100$ .

izes to the Golgi complex. The images of subcellular location of GM130, a *cis*-Golgi protein, showed a similar pattern as PMEPA1-GFP fusion protein (Fig. 2B). Superimposition of the images of PMEPA1-GFP fusion protein and GM130 in LNCaP-PMEPA1-GFP transfectants confirmed the localization of PMEPA1-GFP fusion protein on *cis*-Golgi structure (Fig. 2C). We did not see the colocalization of PMEPA1-GFP and TGN-38, which localizes to TGN (data not shown). In this regard, the subcellular localization of *PMEPA1* is similar to other two newly identified Nedd4 WW domain-binding proteins, N4wbp5 and N4wbp5a, which were also localized to the



**Fig. 3. Effect of PMEPA1 on colony formation.** Prostate tumor cell lines C4 (A), C4-2 (B), C4-2B (C), LNCaP (panel D), DU145 (E), and PC3 (F) were transfected with 3  $\mu$ g each of PMEPA1-V5-pcDNA3.1 (PMEPA1) and pcDNA3.1 vector (Vector) in triplicate wells. In separate experiments, LNCaP (G) and PC3 (H) cells were transfected with control vector or expression vectors encoding wt-PMEPA1 or PMEPA1-PY mutants (1, PMEPA1-V5-pcDNA3.1; 2, PMEPA1-PY1m-pcDNA3.1; 3, PMEPA1-PY2m-pcDNA3.1; 4, PMEPA1-PY1m/PY2m-pcDNA3.1; 5, pcDNA3.1). Transfected cells were selected for plasmid-containing cells with G418 for 3 weeks, and surviving cells were fixed and stained with crystal violet. Colonies were counted and displayed as histograms. For each cell line, a photograph of one dish of cells treated with 3  $\mu$ g of each plasmid is also shown.



**Fig. 4. Effect of PMEPA1 on cell proliferation.** Stable PMEPA1-GFP-Tet-LNCaP transfectants (described in "Materials and Methods") were seeded in 96-well plates with or without 1  $\mu$ g/ml tetracycline in the medium. The cell proliferation was measured using the CellTiter 96 Aqueous One Solution kit at the indicated time. Tet+ and Tet-, the cell culture medium with or without tetracycline, respectively. The absorbance values which reflected the cell numbers are significantly different ( $P < 0.01$ ) between the two groups, except on day 1.

**Table 1 PMEPA1 expression status in primary prostate cancers**

*PMEPA1* gene expression in 62 matched LCM-derived prostatic cancer cells (T) and normal cells (N) was analyzed by QRT-PCR. The results were represented as differential expression in tumor tissue, defined as 1- (1.5-3-fold), 2- (3.1-10-fold), 3- (10.1-20-fold), and 4- (>20-fold) decreased expression as compared with matched normal tissue; T > N, overexpression in tumor tissue, defined as 1+ (1.5-3-fold), 2+ (3.1-10-fold), 3+ (10.1-20-fold), and 4+ (>20-fold) increased expression as compared with matched normal tissue; and T = N, similar expression between T and N as <1.5-fold (0).

<i>PMEPA1</i> expression	No. of patients/group (%)	Degree of <i>PMEPA1</i> expression	
		Quantity	No. (%)
T < N	40 (64.5)	1- 11 (27.5) 2- 17 (42.5) 3- 5 (12.5)	
T > N	10 (16.1)	4- 7 (17.5) 1+ 6 (60.0) 2+ 4 (40.0) 3+ 0	
T = N	12 (19.4)	4+ 0	

Golgi complex (12, 17). N4wbp5a was observed to sequester the trafficking of Nedd4/Nedd4-2, thereby increasing the activity of NEDD4 (12). As a highly ARG and NEDD4-binding protein, the localization of PMEPA1 on Golgi apparatus may suggest for the role of PMEPA1 in the regulation of protein turnover of AR targets.

**PMEPA1 Inhibits Growth of CaP Cells.** To investigate the biological effects of PMEPA1 expression in the regulation of cell growth and contribution of PY motifs for such functions, we performed the colony formation assay by transfection expression vectors of the wt-PMEPA1 and PMEPA1-PY mutants. As shown in Fig. 3, A-F, colony-forming abilities of CaP cell lines DU145, PC3, LNCaP, and LNCaP sublines were significantly suppressed by transfection of the sense version of wt-PMEPA1 expression vector. Under these conditions, wt-pS3 showed similar cell growth inhibition (data not shown). In two independent experiments, mutations of PY1 motif appear to abolish the inhibition of colony formation by wt-PMEPA1, emphasizing the role of PY1 motif in PMEPA1 and NEDD4 interactions and the biological functions of PMEPA1 (Fig. 3, G and H). It is intriguing that both PY1 and PY2 mutations diminished the *in vitro* binding of PMEPA1 to NEDD4; however, in the context of biological function of PMEPA1 in the colony-forming assay, only mutation of PY1 motif has functional consequence. The growth inhibitory effect of PMEPA1 appears to be linked to the interactions of PY1 motif to NEDD4 WW

PMEPA1 INHIBITS PROSTATE CANCER CELL GROWTH

Table 2 PMEPA1 expression and clinicopathological features

The patients were grouped into two groups: T < N ( $n = 40$ ), PMEPA1 expression reduced  $\geq 1.5$ -fold in tumor cells in comparison with matched normal cells, and T  $\geq N$ , PMEPA1 expression was similar between tumor and normal cells ( $n = 12$ ) or increased in tumor cells ( $n = 10$ ).

PMEPA1 expression	Pathologic stage (%)		PSA range (%)		PSA recurrence (%)		Time to recurrence after surgery (month)
T < N	T2 11 (27.5)	T3 29 (72.5)	$\leq 4$ ng/ml 1 (2.5)	4.1–10 ng/ml 30 (75.0)	10.1–20 ng/ml 9 (22.5)	No 29 (72.5)	Mean $\pm$ SE 8.2 $\pm$ 3.4
T $\geq N$	12 (54.5)	10 (45.5)	5 (22.7)	15 (68.2)	2 (9.1)	Yes 11 (27.5)	18.4 $\pm$ 6.3
P	0.035			0.023		3 (13.6)	0.18
						0.211	

domain. This interpretation is based on the striking observations showing distinctively more colonies with PY1 motif mutant in comparison with wt-PMEPA1. Moreover, the growth inhibitory effect of PMEPA1 has been confirmed by the cell proliferation characteristics of stable PMEPA1-GFP-Tet-LNCaP cells, where exogenous PMEPA1 is up-regulated in the absence of tetracycline. The growth of the PMEPA1-GFP-Tet LNCaP cells in tetracycline-negative medium is significantly slower than that of PMEPA1-tet LNCaP transfector in tetracycline-positive medium (Fig. 4). LNCaP cells with PMEPA1 overexpression also revealed increased retinoblastoma phosphorylation, further confirming cell growth inhibitory effect of PMEPA1 (data not shown).

PMEPA1 is expressed in AR-positive CaP cell lines: LNCaP and its sublines (C4, C4-2, and C4-2B). LNCaP cells are androgen dependent for growth. Although the growth of LNCaP sublines is androgen independent, AR is critical for their proliferation (24). We observed that overexpression of PMEPA1 by transfecting the PMEPA1 expression vector into LNCaP and its sublines significantly inhibited the cell proliferation. Because our preliminary observations showed that PMEPA1 overexpression in LNCaP cells resulted in altered expression of AR downstream genes,<sup>5</sup> we hypothesized that the growth inhibitory effect of PMEPA1 on LNCaP and its sublines may be mediated through directly or indirectly affecting AR functions. Despite the growth inhibitory effect on AR-positive CaP cell lines, PMEPA1 was also found to inhibit the growth of AR-negative prostate tumor cells, DU145 and PC3, suggesting that the growth inhibitory effects of PMEPA1 on DU145 and PC3 could be mediated through alternative mechanisms, e.g., regulation of other nuclear steroid receptors by PMEPA1. Nonetheless, inhibition of CaP cell growth by PMEPA1 suggested that PMEPA1 might be involved in CaP development.

**Decreased PMEPA1 Expression in Prostate Tumor Tissues.** CaP cell growth inhibitory functions of PMEPA1 led us to carefully evaluate the relationship of PMEPA1 expression alterations to the clinicopathologic features of CaP. The overall expression pattern of the PMEPA1 is shown in Table 1. Comparison of PMEPA1 expression between tumor and normal cells revealed tumor cell-associated decreased expression (T < N) in 64.5% tumor specimens (40 of 62), increased expression (T > N) in 16.1% specimens (10 of 62), and no change (T = N) in 19.4% specimens (12 of 62). When these expression patterns were stratified by organ-confined (pT2) and nonorgan-confined (pT3) disease, a higher percentage of PMEPA1 reduction was seen in pT3 (74%) versus pT2 (48%). Because the T > N group has a few cases, we combined the T > N and T = N groups (T  $\geq$  N group). Comparison of the clinicopathologic parameters between the PMEPA1-T < N and PMEPA1-T  $\geq$  N groups revealed that the PMEPA1-T < N group had a significantly higher percentage of patients with pT3 tumors ( $P = 0.035$ ), and more patients in this group had a higher level of preoperative serum PSA ( $P = 0.023$ ; Table 2). Of 62 patients whose tumors were analyzed for PMEPA1 expression, 14 patients showed CaP recurrence as defined by serum PSA

level  $\geq 0.2$  ng/ml after prostatectomy. Of the 14 patients, 11 showed reduced tumor-associated PMEPA1 expression (78.5%). Reduced PMEPA1 expression seems to associate with a higher recurrence rate and shorter duration to recurrence after surgery, although the statistical analysis did not reveal a significant difference which might be attributable to the small number of patients (Table 2). It is worth noting these observations in the context of the report showing consistent high levels of induction of PMEPA1 in nontumorigenic revertants of tumor cell lines after the transfer of human chromosome 8p (18), which harbors putative TSGs (25, 26). Among chromosomal "hot spots" in CaP and other cancers, 8p loss is particularly frequent, occurring in ~80% of prostate tumors (27–29). Transfer of single human chromosome 8 has been shown to result in suppression of the malignant phenotype or the metastatic ability in a variety of cell lines (18, 30, 31). Therefore, it is possible that the reduced expression of PMEPA1 in prostate tumors might result from the loss of the putative TSGs or cell growth inhibitory genes on chromosome 8p in prostate tumor cells. Two very recent interesting reports have described PMEPA1 as a transforming growth factor- $\beta$ -induced gene and marker of terminal colonocyte differentiation (32, 33).

In summary, we have demonstrated that PMEPA1 interacts with NEDD4 through its PY motifs. PMEPA1 has cell growth inhibitory effects when overexpressed in androgen-dependent or -independent CaP cells. PY1 motif of PMEPA1 appears to modulate its cell growth inhibitory function. PMEPA1 may function in the regulation of protein turnover via ubiquitination/proteasome pathways in cells. The biological effects of PMEPA1 in CaP cells and expression pattern of PMEPA1 in CaP indicate that PMEPA1 may function as the cell growth inhibitor regulated by androgen.

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#### References

- Coffey, D. S. The molecular biology, endocrinology and physiology of the prostate and seminal vesicle. In: P. C. Walsh, A. B. Retik, T. A. Stamey, and E. D. Vaughan, Jr. (eds.), *Campbell's Urology*, Vol. 1, pp. 221–266. Philadelphia: WB Saunders, 1992.
- Xu, L., Su, Y., LaBiche, R., Segawa, T., Shammugam, N., McLeod, D. G., Moul, J. W., and Srivastava, S. Quantitative expression profile of androgen regulated genes (ARGs) in prostate cancer cells and identification of prostate specific genes. *Int. J. Cancer*, 92: 322–328, 2001.
- Xu, L. L., Shammugam, N., Segawa, T., Sesterhenn, I. A., McLeod, D. G., Moul, J. W., and Srivastava, S. A novel androgen-regulated gene, PMEPA1, located on chromosome 20q13 exhibits high level expression in prostate. *Genomics*, 66: 257–263, 2000.
- Jolliffe, C. N., Harvey, K. F., Haines, B. P., Parasivam, G., and Kumar, S. Identification of multiple proteins expressed in murine embryos as binding partners for the WW domains of the ubiquitin-protein ligase Nedd4. *Biochem. J.*, 351: 557–565, 2000.
- Kumar, S., Tomooka, Y., and Noda, M. Identification of a set of genes with developmentally down-regulated expression in the mouse brain. *Biochem. Biophys. Res. Commun.*, 185: 1155–1161, 1992.
- Kumar, S., Harvey, K. F., Kinoshita, M., Copeland, N. G., Noda, M., and Jenkins, N. A. CDNA cloning, expression analysis, and mapping of the mouse Nedd4 gene. *Genomics*, 40: 435–443, 1997.
- Anan, T., Nagata, Y., Koga, H., Honda, Y., Yabuki, N., Miyamoto, C., Kuwano, A., Matsuda, I., Endo, F., Saya, H., and Nakao, M. Human ubiquitin-protein ligase Nedd4: expression, subcellular localization and selective interaction with ubiquitin-conjugating enzymes. *Gene Cells*, 3: 751–763, 1998.

<sup>5</sup> L. L. Xu *et al.*, unpublished data.

8. Harvey, K. F., and Kumar, S. Nedd4-like proteins: an emerging family of ubiquitin-protein ligases implicated in diverse cellular functions. *Trends Cell Biol.*, **9**: 166–169, 1999.
9. Hicke, L. A new ticket for entry into budding vesicles-ubiquitin. *Cell*, **106**: 527–530, 2001.
10. Goulet, C. C., Volk, K. A., Adams, C. M., Prince, L. S., Stokes, J. B., and Snyder, P. M. Inhibition of the epithelial Na<sup>+</sup> channel by interaction of Nedd4 with a PY motif deleted in Liddle's syndrome. *J. Biol. Chem.*, **273**: 30012–30017, 1998.
11. Pham, N., and Rotin, D. Nedd4 regulates ubiquitination and stability of the guanine-nucleotide exchange factor CNrasGEF. *J. Biol. Chem.*, **276**: 46995–47003, 2001.
12. Konstas, A. A., Shearwin-Whyatt, L. M., Fotia, A. B., Degger, B., Riccardi, D., Cook, D. I., Korbmacher, C., and Kumar, S. Regulation of the epithelial sodium channel by N4WPBP5A, a novel Nedd4/Nedd4-2-interacting protein. *J. Biol. Chem.*, **277**: 29406–29416, 2002.
13. Sudol, M. The WW module competes with the SH3 domain? *Trends Biochem. Sci.*, **21**: 161–163, 1996.
14. Macias, M. J., Hyvonen, M., Baraldi, E., Schultz, J., Sudol, M., Saraste, M., and Oschkinat, H. Structure of the WW domain of a kinase-associated protein complexed with a proline-rich peptide. *Nature (Lond.)*, **382**: 646–649, 1996.
15. Chen, H. I., and Sudol, M. The WW domain of Yes-associated protein binds a proline-rich ligand that differs from the consensus established for Src homology 3-binding modules. *Proc. Natl. Acad. Sci. USA*, **92**: 7819–7823, 1995.
16. Murillas, R., Simms, K. S., Hatakeyama, S., Weissman, A. M., and Kuehn, M. R. Identification of developmentally expressed proteins that functionally interact with Nedd4 ubiquitin ligase. *J. Biol. Chem.*, **277**: 2897–2907, 2002.
17. Harvey, K. F., Shearwin-Whyatt, L. M., Fotia, A., Parton, R. G., and Kumar, S. N4WPBP5, a potential target for ubiquitination by the Nedd4 family of proteins, is a novel Golgi-associated protein. *J. Biol. Chem.*, **277**: 9307–9317, 2002.
18. Banerjee, K., Arbieva, H., Usha, L., Le, T. T., Liang, J., Gomes, I., and Westbrook, C. A. Identification of downstream targets of the putative tumor suppressor gene on 8p by differential gene expression analysis. AACR Annual meeting, Proceedings, **42**: 428, 2001.
19. Rae, F. K., Hooper, J. D., Nicol, D. L., and Clements, J. A. Characterization of a novel gene, STAG1/PMEPA1, upregulated in renal cell carcinoma and other solid tumors. *Mol. Carcinog.*, **32**: 44–53, 2001.
20. Hsieh, J. T., Wu, H. C., Gleave, M. E., von Eschenbach, A. C., and Chung, L. W. Autocrine regulation of prostate-specific antigen gene expression in a human prostatic cancer (LNCaP) subline. *Cancer Res.*, **53**: 2852–2857, 1993.
21. Thalmann, G. N., Anczisin, P. E., Chang, S. M., Zhai, H. E., Kim, E. E., Hopwood, V. L., Pathak, S., von Eschenbach, A. C., and Chung, L. W. Androgen-independent cancer progression and bone metastasis in the LNCaP model of human prostate cancer. *Cancer Res.*, **54**: 2577–2581, 1994.
22. Wu, T. T., Sikes, R. A., Cui, Q., Thalmann, G. N., Kao, C., Murphy, C. F., Yang, H., Zhai, H. E., Balian, G., and Chung, L. W. Establishing human prostate cancer cell xenografts in bone: induction of osteoblastic reaction by prostate-specific antigen-producing tumors in athymic and SCID/bg mice using LNCaP and lineage-derived metastatic sublines. *Int. J. Cancer*, **77**: 887–894, 1998.
23. Xu, L. L., Stackhouse, B. G., Florence, K., Zhang, W., Shanmugam, N., Sesterhenn, I. A., Zou, Z., Srikanthan, V., Augustus, M., Roschke, V., Carter, K., McLcod, D. G., Moul, J. W., Soppett, D., and Srivastava, S. PSGR, a novel prostate-specific gene with homology to a G protein-coupled receptor, is overexpressed in prostate cancer. *Cancer Res.*, **60**: 6568–6572, 2000.
24. Zegarra-Moro, O. L., Schmidt, L. J., Huang, H., and Tindall, D. J. Disruption of androgen receptor function inhibits proliferation of androgen-refractory prostate cancer cells. *Cancer Res.*, **62**: 1008–1013, 2002.
25. Bookstein, R., Bova, G. S., MacGrogan, D., Levy, A., and Isaacs, W. B. Tumour-suppressor genes in prostatic oncogenesis: a positional approach. *Br. J. Urol.*, **79** (Suppl. 1): 28–36, 1997.
26. Isaacs, W., and Kainu, T. Oncogenes and tumor suppressor genes in prostate cancer. *Epidemiol. Rev.*, **23**: 36–41, 2001.
27. Bova, G. S., Carter, B. S., Bussemakers, M. J., Emi, M., Fujiwara, Y., Kyprianou, N., Jacobs, S. C., Robinson, J. C., Epstein, J. I., Walsh, P. C., et al. Homozygous deletion and frequent allelic loss of chromosome 8p22 loci in human prostate cancer. *Cancer Res.*, **53**: 3869–3873, 1993.
28. Emmert-Buck, M. R., Vocke, C. D., Pozzatti, R. O., Duray, P. H., Jennings, S. B., Florence, C. D., Zhuang, Z., Bostwick, D. G., Liotta, L. A., and Linehan, W. M. Allelic loss on chromosome 8p12–21 in microdissected prostatic intraepithelial neoplasia. *Cancer Res.*, **55**: 2959–2962, 1995.
29. Swalwell, J. I., Vocke, C. D., Yang, Y., Walker, J. R., Grouse, L., Myers, S. H., Gillespie, J. W., Bostwick, D. G., Duray, P. H., Linehan, W. M., and Emmert-Buck, M. R. Determination of a minimal deletion interval on chromosome band 8p21 in sporadic prostate cancer. *Genes Chromosomes Cancer*, **33**: 201–205, 2002.
30. Nihei, N., Ichikawa, T., Kawana, Y., Kuramochi, H., Kugoh, H., Oshimura, M., Hayata, I., Shimazaki, J., and Ito, H. Mapping of metastasis suppressor gene(s) for rat prostate cancer on the short arm of human chromosome 8 by irradiated microcell-mediated chromosome transfer. *Genes Chromosomes Cancer*, **17**: 260–268, 1996.
31. Gustafson, C. E., Wilson, P. J., Lukeis, R., Baker, E., Woollett, E., Annab, L., Hawke, L., Barrett, J. C., and Chenevix-Trench, G. Functional evidence for a colorectal cancer tumor suppressor gene at chromosome 8p22–23 by monochromosome transfer. *Cancer Res.*, **56**: 5238–5245, 1996.
32. Brunschwig, E. B., Wilson, K., Mack, D., Dawson, D., Lawrence, E., Willson, J. K., Lu, S., Nosrati, A., Rekro, R. M., Swintler, S., Beard, L., Lutterbaugh, J. D., Willis, J., Platzer, P., and Markowitz, S. PMEPA1, a transforming growth factor-β-induced marker of terminal colonocyte differentiation whose expression is maintained in primary and metastatic colon cancer. *Cancer Res.*, **63**: 1568–1575, 2003.
33. Itoh, S., Thorikay, M., Kowanetz, M., Moustakas, A., Itoh, F., Heldin, C. H., and ten Dijke, P. Elucidation of Smad requirement in transforming growth factor-beta type I receptor-induced responses. *J. Biol. Chem.*, **278**: 3751–3761, 2003.

# Characterization of a Novel Gene, *STAG1/PMEPA1*, Upregulated in Renal Cell Carcinoma and Other Solid Tumors

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Using differential display–polymerase chain reaction, we identified a novel gene sequence, designated solid tumor-associated gene 1 (*STAG1*), that is upregulated in renal cell carcinoma (RCC). The full-length cDNA (4839 bp) encompassed the recently reported androgen-regulated prostatic cDNA *PMEPA1*, and so we refer to this gene as *STAG1/PMEPA1*. Two *STAG1/PMEPA1* mRNA transcripts of approximately 2.7 and 5 kb, with identical coding regions but variant 3' untranslated regions, were predominantly expressed in normal prostate tissue and at lower levels in the ovary. The expression of this gene was upregulated in 87% of RCC samples and also was upregulated in stomach and rectal adenocarcinomas. In contrast, *STAG1/PMEPA1* expression was barely detectable in leukemia and lymphoma samples. Analysis of expressed sequence tag databases showed that *STAG1/PMEPA1* also was expressed in pancreatic, endometrial, and prostatic adenocarcinomas. The *STAG1/PMEPA1* cDNA encodes a 287-amino-acid protein containing a putative transmembrane domain and motifs that suggest that it may bind src homology 3– and tryptophan tryptophan domain-containing proteins. This protein shows 67% identity to the protein encoded by the chromosome 18 open reading frame 1 gene. Translation of *STAG1/PMEPA1* mRNA in vitro showed two products of 36 and 39 kDa, respectively, suggesting that translation may initiate at more than one site. Comparison to genomic clones showed that *STAG1/PMEPA1* was located on chromosome 20q13 between microsatellite markers *D20S183* and *D20S173* and spanned four exons and three introns. The upregulation of this gene in several solid tumors indicated that it may play an important role in tumorigenesis. © 2001 Wiley-Liss, Inc.

**Key words:** differential display; polymerase chain reaction; 20q13; gene expression

## INTRODUCTION

Tumorigenesis is the result of many genetic alterations, which act coordinately to contribute to the disease process. Identification of the molecular events of tumor progression is crucial to providing targets for cancer prevention and treatment. Such techniques as differential display (DD)–polymerase chain reaction (PCR) [1] are valuable tools for isolating disease-associated genes. For example, DD-PCR has been used extensively to identify genes that are expressed differentially in a range of cancers, including breast, prostate, and ovarian cancer [2–4]. Although a number of genes associated with renal cell carcinoma (RCC) have been identified using DD-PCR [5–8], their precise role in RCC tumorigenesis is yet to be elucidated.

To identify additional genes that are expressed differentially in RCC, DD-PCR was performed to compare RNA derived from RCC with RNA from normal kidney parenchyma obtained from the same person. Using this approach, we pinpointed 14 genes differentially expressed in RCC [9]. One of these novel partial gene sequences was upregulated in RCC and was chosen for further analysis. In this study, we further characterized this gene. The full-length cDNA encompassed the recently reported

androgen-regulated prostatic *PMEPA1* cDNA [10]. We showed that this gene not only was overexpressed in RCC but also was upregulated in other solid tumors. Because our analyses indicated that there was virtually no expression in leukemia and lymphoma samples, we termed this gene solid tumor–associated gene 1 (*STAG1*), and we now suggest that a more appropriate name may be *STAG1/PMEPA1*.

## MATERIALS AND METHODS

### Clinical Samples

Kidney tissue was collected at the time of nephrectomy from patients with RCC at the Princess

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Abbreviations: DD, differential display; PCR, polymerase chain reaction; RCC, renal cell carcinoma; *STAG1*, solid tumor–associated gene 1; nr, nonredundant; EST, expressed sequence tag; RT, reverse transcription; UTR, untranslated region; C18orf1, chromosome 18 open reading frame 1; SH3, src homology 3; WW, tryptophan tryptophan.

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Alexandra Hospital, Brisbane, Australia, and stored at -80°C. Histologic assessment of tumor tissue from the 16 patients participating in this study confirmed clear cell RCC, based on the Heidelberg classification of renal cell tumors [11], with a tumor stage of T1 or T2, N0, M0 (stage I or II), as determined by the tumor, node, metastasis (TNM) staging of RCC [12]. Informed consent was obtained in all cases. Ethics approval was obtained from both the Queensland University of Technology and Princess Alexandra Hospital ethics committees.

#### DD-PCR, Identification, and Characterization of the STAG1/PMEPA1 cDNA and Gene

DD-PCR was performed using a Delta Differential Display kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol, with modifications as described previously [9,13]. Duplicate paired RCC and normal kidney samples from four different patients were analyzed using 65 different primer combinations. A 434-bp cDNA, identified as being upregulated in all four patient's RCC samples, was cloned into pGEM-T Easy (Promega, Madison, WI) and sequenced. This sequence then was used to screen the National Centre for Biotechnology Information GenBank nonredundant (nr) and expressed sequence tag (EST) databases. Matching clones (accession nos. AI446289, AI521341, AI587542, AI677810, and AI972096) were purchased (Incyte Genomics, Palo Alto, CA) and sequenced. Additional sequencing, to generate a complete contig, was obtained by PCR using the following primers: 5'-GGATAAACAGAAAGGACACCC-3' (forward) and 5'-AGGCGACTCTGAAATTCCG-3' (reverse), after reverse transcription (RT) of oligo(dT)-primed RCC total RNA.

#### In Vitro Transcription and Translation

Two templates, for use in in vitro transcription/translation experiments, were generated by PCR from EST clone AI677810 using forward primers 5'-GGATCTTAATACGACTCACTATAGGGAGACCAC-CATCGTCCATGCACCGCTTGATGG -3' and 5'-GGATCTTAATACGACTCACTATAGGGAGACCAC-CATGTCCAGAGCATGGAGATCAG-3' (T7 promoter sequence underlined) and reverse primer 5'-GGACCTAGAGAGGGTGTCC-3'. The fidelity of these PCR products was confirmed by sequencing. Each PCR product (1 µg) was transcribed and translated in vitro using a TNT T7 Coupled Reticulocyte Lysate System (Promega) in the presence of <sup>35</sup>S-methionine (Amersham, Piscataway, NJ). Control reactions were performed using luciferase cDNA and no DNA. Protein products were separated by electrophoresis on a 12.5% Tris polyacrylamide gel. The gel was fixed, washed in Amplify Reagent (Amersham), dried overnight, and exposed to X-ray film (AGFA Curix, Brisbane, Australia) for 2 h.

#### Northern Analysis

RCC and paired normal kidney total RNAs (20 µg) were separated by denaturing gel electrophoresis and transferred to a Hybond N+ nylon membrane (Amersham). Human Multiple Tissue Northern blots and Matched Tumor/Normal Expression arrays were purchased from Clontech. STAG1/PMEPA1 cDNA (nucleotides 4429–4763), cloned into pGEM-T Easy (Promega), was linearized with SalI before transcription with T7 RNA polymerase, generating an antisense [ $\alpha$ -<sup>32</sup>P]UTP-labeled cRNA probe using a StripEz RNA kit (Ambion, Austin, TX). Hybridizations were performed at 68°C overnight in Ultrahyb solution (Ambion) and washed to a final stringency of 0.1× sodium chloride, sodium citrate/0.1% sodium dodecyl sulfate at 72°C. The Human Multiple Tissue Northern blots and the Matched Tumor/Normal array also were probed with a cRNA probe generated from EST clone AI972096 (nucleotides 1–1403 of the STAG1/PMEPA1 cDNA), which had been linearized with SalI. Blots were reprobed with  $\beta$ -actin cDNA to confirm RNA loadings.

#### Semiquantitative RT-PCR

Semiquantitative RT-PCR was performed on RCC and matching normal kidney tissue from 12 patients (six males and six females), using the following PCR primers: 5'-CCAGAACCATAAAAAT-ATCCCG-3' and 5'-CAACAAGCAGTTCTTCAGGCC-3'. Optimal cycling parameters, shown to be in the linear range of amplification, were 94°C for 1 min, 59°C for 1 min, and 72°C for 1 min for 26 cycles, followed by a 7-min extension at 72°C. A control PCR also was performed for  $\beta_2$ -microglobulin for 25 cycles.

## RESULTS

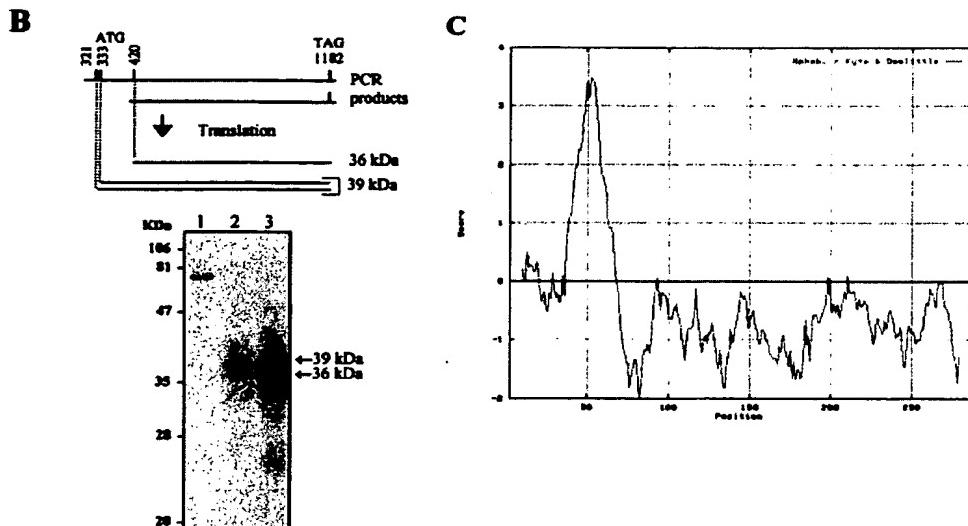
#### Identification and Characterization of STAG1/PMEPA1 cDNA and Protein

Using a modified DD-PCR technique, a novel 434-bp cDNA (Figure 1A), subsequently designated STAG1, was identified as being upregulated in RCC (data not shown). This partial cDNA, which showed no homology to any known genes, contained a polyadenylation signal consensus sequence (AATAAA) [14]. An additional sequence was obtained from clones identified by searching the EST database. The complete 5' (nucleotides 1–1659) and 3' (nucleotides 1848–4839) ends of the cDNA were obtained from clones AI677810 and AI446289, respectively. Sequence spanning these clones (nucleotides 1156–1899) was amplified from RCC total RNA by RT-PCR. From these sequences, a 4839-bp contig (Figure 1A) of the STAG1 cDNA sequence was generated and submitted to GenBank under the accession no. AF305616.

The complete cDNA contained 320 bp of 5' untranslated region (UTR) and a 3' UTR of 3658 bp,

A

Figure 1. (Continued next page)



**Figure 1.** (A) Nucleotide and amino acid sequence of human STAG1/PMEPA1. Nucleotides are numbered on the left and amino acids on the right. Exon/intron boundaries are marked with arrowheads. Three closely spaced start codons at positions 321–323, 333–335, and 420–422 are underlined with solid lines, and consensus polyadenylation signals at positions 2158, 2463, and 4818 are underlined with two solid lines. The PMEPA1 nucleotide sequence [10] is marked in bold, and the STAG1 fragment identified by DD-PCR is marked by broken underlining. The potential transmembrane domain is boxed in gray. Consensus  $N$ -glycosylation sites at  $N^9$ ,  $N^{19}$ , and  $N^{188}$  are marked by wavy underlining in the protein sequence. (B) In vitro transcription/translation of STAG1/PMEPA1 cDNA. Schematic representation of the PCR products used for in vitro transcription/translation and the protein products generated. Lane 1,

control reaction from luciferase cDNA, giving a protein product of the predicted size (61 kDa); lane 2, proteins generated from the PCR product, containing nucleotides 313–1189 of the STAG1/PMEPA1 cDNA; lane 3, protein generated from the PCR product, containing nucleotides 413–1189 of the STAG1/PMEPA1 cDNA. A negative control reaction lacking DNA also was performed and was clear of reaction products (data not shown). Molecular weights are indicated. (C) Hydrophobicity plot of the deduced STAG1/PMEPA1 protein. The method of Kyte and Doolittle [16] was used with a window of 17 residues. The peak corresponds to amino acids 41–62 and represents the putative transmembrane domain. [Figure can be viewed in color in the online version of this article, available at [www.interscience.wiley.com](http://www.interscience.wiley.com)]

with the largest open reading frame of 861 bp (nucleotides 321–1181), and encoded a 287-amino-acid protein of 32 kDa. Consensus polyadenylation signals (AATAAA) were located at nucleotides 2158, 2463, and 4818 (Figure 1A). The STAG1 cDNA encompassed 1042 nucleotides (nucleotides 430–1458) of the recently reported PMEPA1 cDNA [10] (Figure 1A), and therefore this gene will be referred to as STAG1/PMEPA1. This gene also showed homology (77%), at the nucleotide level, over the coding region of chromosome 18 open reading frame 1 (*C18orf1*) cDNA [15].

The STAG1/PMEPA1 cDNA contained three closely spaced methionine codons (Figure 1A), which may be capable of functioning as sites for translation initiation. In vitro transcription/translation from two PCR products, containing nucleotides 313–1189 and 413–1189 of the STAG1/PMEPA1 cDNA, was performed to determine which of the first/second or third ATG codons would be functional in an in vitro system. In vitro transcription/translation from the larger PCR product generated two protein products of approximately 36 and 39 kDa, whereas the shorter PCR product generated a single protein of approximately 36 kDa (Figure 1B).

These data indicated that the ATG codons at 321–323/333–335 and 420–422 are capable of functioning as initiating methionines in vitro. It was not possible to determine from these experiments whether translation from the first or second or both of these ATG codons generated the 39-kDa protein.

To gain insight into the potential role of STAG1/PMEPA1, the deduced protein sequence was analyzed for cellular sorting signals, potential functional and structural domains, and sequence homologies. This analysis indicated that STAG1/PMEPA1 lacked consensus signals for both secretion and translocation to the nucleus. However, identification of a hydrophobic region spanning amino acids 41–62 [16], which had the characteristics of a type Ib transmembrane domain (Figure 1C), indicated that STAG1/PMEPA1 was a putative transmembrane protein with the amino terminus located extracellularly and the carboxy terminus located intracellularly. Although a search of the PROSITE database [17] showed that the deduced STAG1/PMEPA1 protein does not contain any known functional or structural domains, several proline-rich motifs were identified that may function as

binding sites for src homology 3 (SH3) and tryptophan tryptophan (WW) domains [18]. The motifs PPRP (amino acid 112–115), PTYP (amino acid 135–138), and PCPP (amino acid 205–208) conform to the consensus for binding of the SH3 domains (PXXP). In addition, the motifs PPPY (amino acid 158–161) and PPTY (amino acid 229–232) correspond to WW domain consensus binding sites. Furthermore, five putative casein kinase II (amino acids 37, 151, 190, 231, and 254) and four potential protein kinase C (amino acids 68, 116, 182, and 199) phosphorylation sites were detected, along with three consensus motifs for N-linked glycosylation (N-X-T/S) at N<sup>8</sup>, N<sup>19</sup>, and N<sup>188</sup> (Figure 1A) and two potential N-myristoylation sites (amino acids 6 and 213). Searches of the GenBank nr database indicated that *STAG1/PMEPA1* protein showed significant homology (67%) only to the C18orf1 protein.

#### Genomic Structure of *STAG1/PMEPA1*

The *STAG1/PMEPA1* gene sequence was contained within the genomic clones RPS-1059L7, RPS-1007E6, and RP4-718J7 (accession nos. AL121913, AL161943, and AL035541, respectively), which were identified by screening the GenBank nr database (Figure 2A). These clones are located on chromosome 20q13 between microsatellite markers D20S183 and D20S173. The *STAG1/PMEPA1* gene was approximately 62 kb in length and contained five exons and three introns. Exons ranged in size from 54 to 4201 bp and introns from 434 to 49 775 bp. Intron/exon junctions were determined by comparison of cDNA and genomic sequences (Figure 2B). All of the intron/exon junctions conform to the GT-AG rule. Interestingly, the *STAG1/PMEPA1* gene contained two alternatively spliced first exons—exon 1A and exon 1B (Figure 2A). The exon 1A described by Xu et al. [10] also was contained within the genomic clone RPS-1059L7 but was 562 bp further upstream from the exon 1 sequence described here (exon 1B). The use of the alternative exon 1A sequence gives rise to a smaller protein, two amino acids shorter than the smallest protein described in Figure 1A (from the third ATG). The *STAG1/PMEPA1* gene sequence, encompassing exon 1B to exon 4, was submitted to Genbank under the accession no. AF305426.

#### *STAG1/PMEPA1* Expression Pattern in Normal and Malignant Tissues

Northern blot analysis of 16 normal human tissues using a cRNA probe containing the coding region of *STAG1/PMEPA1* (nucleotides 1–1403) showed two transcripts of approximately 5 and 2.7 kb that were highly expressed in the prostate, as previously reported [10]. Lower levels were observed in the ovary, and very low expression was noted in heart, lung, skeletal muscle, pancreas, small intestine, and colon (Figure 3A). When probed with a

cRNA probe derived from the 3' UTR of *STAG1/PMEPA1*, only the 5 kb transcript, which showed the same expression pattern, was noted (Figure 3A). Because the *STAG1/PMEPA1* cDNA (4839 bp), together with a polyadenylation tail, was approximately the same size as the 5-kb transcript seen on Northern analysis, it is likely that we have identified the complete *STAG1/PMEPA1* coding sequence. The smaller, 2.7-kb transcript most likely results from use of the polyadenylation signal at nucleotide 2465 (Figure 1A). To extend the expression analysis, a multiple tissue expression array containing polyA+ RNA from 76 normal human tissues and cell lines was probed with the 5' end of the *STAG1/PMEPA1* cRNA probe. Confirming the results of Northern blot analysis, significant levels of *STAG1/PMEPA1* mRNA were detected in the prostate (no. 53) (Figure 3B). Significant levels of expression also were detected in putamen (no. 16), aorta (no. 23), and the lung carcinoma cell line A549 (no. 69) (Figure 3B). While there was moderate expression of *STAG1/PMEPA1* in the majority of tissues arrayed on the blot, there was virtually no expression in the leukemia (nos. 62, 64, and 65) and lymphoma (nos. 66 and 67) samples (Figure 3B).

To confirm the upregulation of this gene in RCC, as shown by DD-PCR, Northern analysis and semiquantitative RT-PCR were performed. Northern analysis of three paired RCC and normal samples showed that *STAG1/PMEPA1* mRNA was detected only in RCC (data not shown). To examine a larger number of samples, semiquantitative RT-PCR was performed on another 12 RCC and normal kidney paired samples. Upregulation of *STAG1/PMEPA1* in RCC was confirmed in 10 of the 12 (83%) paired samples (Figure 3C). Together with the DD-PCR results, *STAG1/PMEPA1* was upregulated in 14 of 16 (87%) paired RCC samples.

Analysis of the source of the ESTs matching the *STAG1/PMEPA1* sequence showed that this gene also was expressed in a wide variety of other malignancies, such as pancreatic, endometrial, stomach, and prostate adenocarcinoma. To determine whether *STAG1/PMEPA1* is upregulated in any other malignancies, we analyzed a matched tumor/normal expression array containing cDNA from 68 tumor and corresponding normal tissues from individual patients with a variety of different cancers (kidney, breast, prostate, uterus, ovary, cervix, colon, lung, stomach, rectum, and small intestine). Confirming our semiquantitative RT-PCR and Northern blot analysis results, 10 of 14 kidney cancers (all clear cell RCCs ranging from stage II to IV) showed more intense hybridization than their normal kidney counterparts (Figure 4A). Seven of eight stomach adenocarcinomas and five of seven rectal adenocarcinomas showed significantly higher levels of expression of *STAG1/PMEPA1* compared with the corresponding normal samples (Figure 4B)

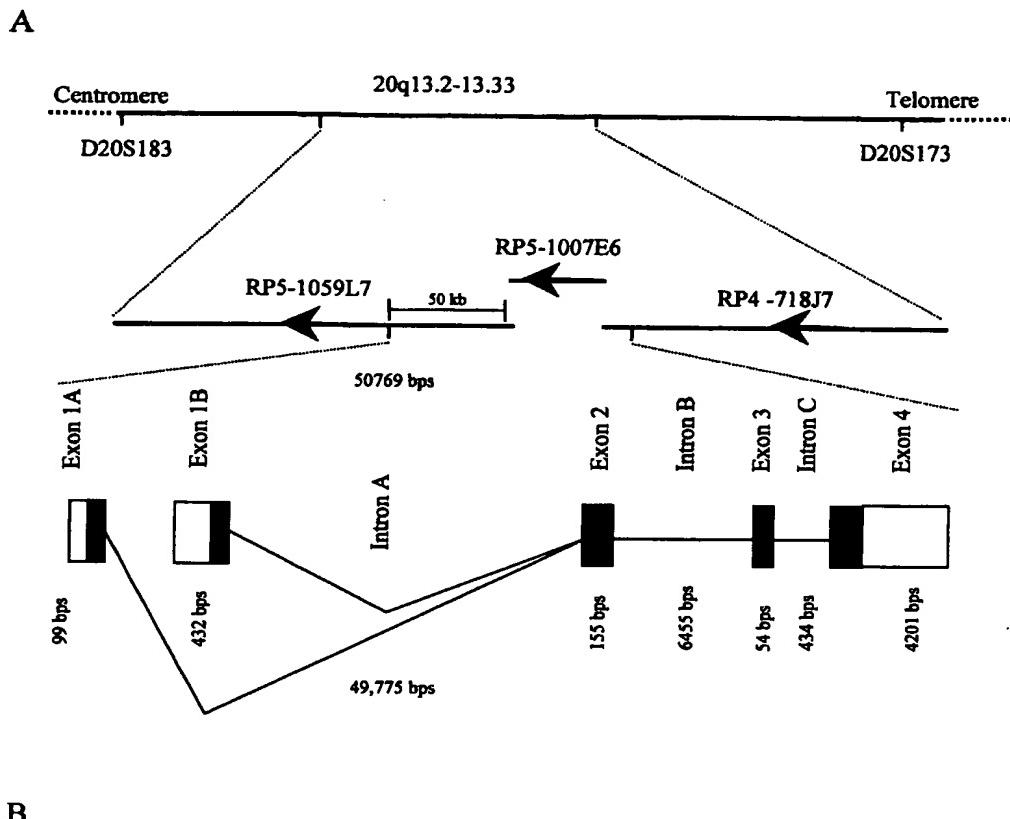


Figure 2. (A) *STAG1/PMEPA1* gene location and structure. The *STAG1/PMEPA1* gene was located on human chromosome 20q13 between microsatellite markers D20S183 and D20S173 and spanned the genomic clones RP5-1059L7, RP5-1007E6, and RP4-718J7 (accession nos. AL121913, AL161943, and AL035541, respectively). Arrows represent the direction of the genomic clones. Exons are represented as filled boxes and untranslated regions as unfilled

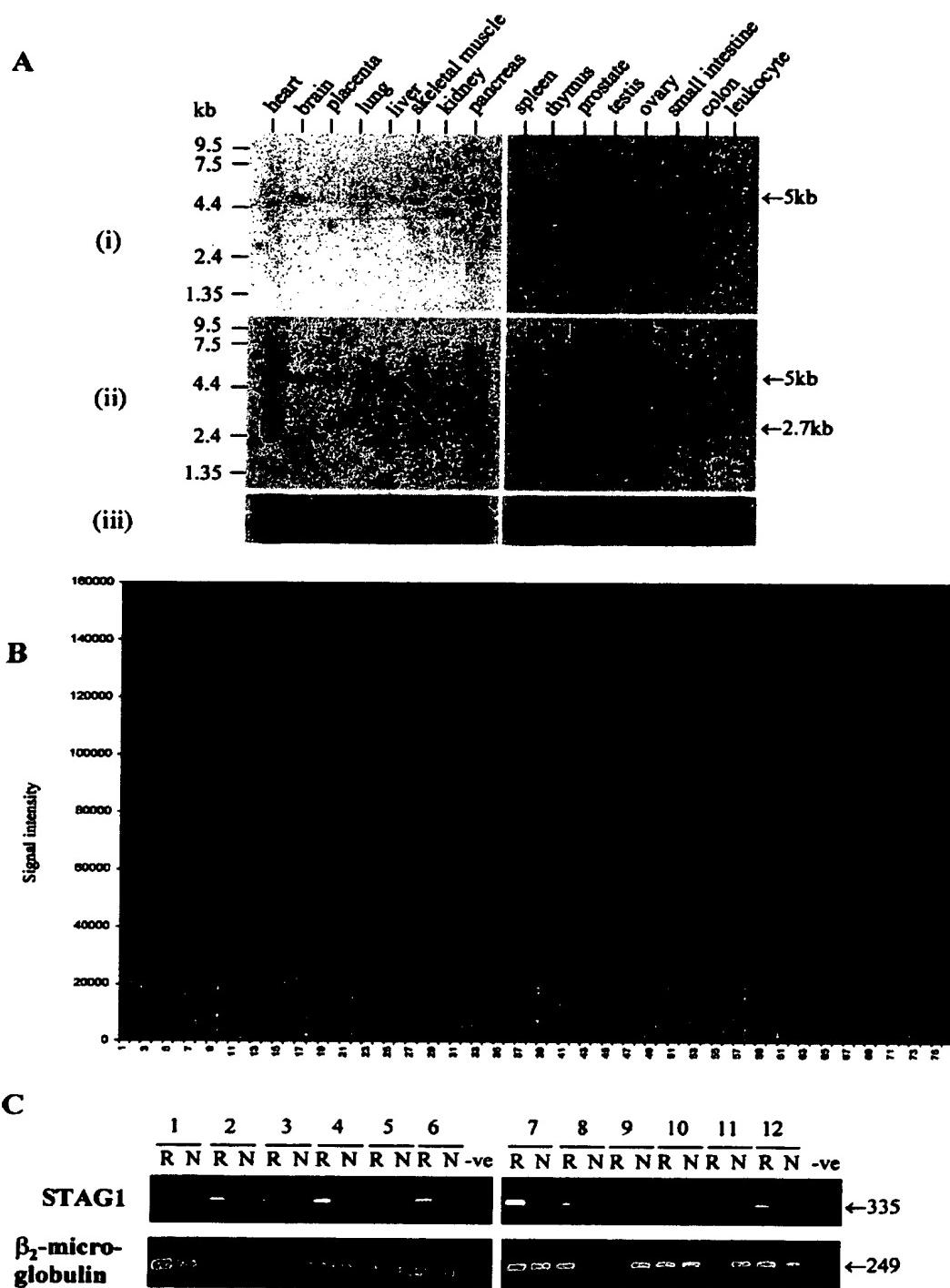
boxes; introns are given as lines (not to scale). Exon and intron sizes are indicated. (B) Exon/intron boundaries of the *STAG1/PMEPA1* gene. Exon and intron sequences are shown in uppercase and lowercase letters, respectively. The nucleotide consensus sequence of the intron adjoining the splice junctions are shown in boldface type.

and C). There was no consistent differential expression patterns in the other paired normal and malignant samples from other tissues.

#### DISCUSSION

To date, a small number of studies have examined RCC using the DD-PCR technique [5-8]. A number of interesting differentially expressed genes have

been identified from these studies, but their precise role in RCC tumorigenesis is yet to be elucidated. Using a modified DD-PCR technique, we showed that a diverse range of genes is upregulated in RCC [9]. Characterization of a novel sequence upregulated in RCC showed that this gene also had increased expression in solid tumors of the stomach and rectum but was not expressed in leukemia and



lymphoma samples and hence was termed *STAG1* (solid tumor – associated gene 1). The full-length cDNA of *STAG1* encompassed the recently described androgen-regulated *PMEPA1* [10], and so we termed this gene *STAG1/PMEPA1*. *STAG1/PMEPA1* encoded a putative transmembrane protein containing potential binding sites for proteins with SH3 and WW domains.

The coding region of *STAG1/PMEPA1* showed 77% homology to another gene, *C18orf1*, which is located on chromosome 18 and encodes several transcript variants predominantly expressed in the brain [15]. Like *STAG1/PMEPA1*, *C18orf1* has a large 3' UTR of 7.1 kb that contains 10 potential polyadenylation sites, a number of which are used to generate multiple transcripts. The 3.6 kb 3' UTR of *STAG1/PMEPA1* contained three consensus polyadenylation signals; only two *STAG1/PMEPA1* transcripts (2.7 and 5 kb) were detected on Northern blot analysis. The polyadenylation signal at 2463 bp most likely was used to generate the 2.7-kb transcript, though analysis of the EST database showed that ESTs were generated from both the polyadenylation signals at nucleotides 2463 and 2158. *STAG1/PMEPA1* also had alternately spliced first exons. Besides sequence similarity, *C18orf1* and *STAG1/PMEPA1* both had a transmembrane domain, and the *STAG1/PMEPA1* protein was of a size similar to the  $\beta 1$  isoform of *C18orf1*. These two genes most likely belong to a novel gene family whose functions remain to be elucidated.

The *STAG1/PMEPA1* cDNA contained three closely spaced potential translation-initiating codons at nucleotides 321–323, 333–335, and 420–422. The ATG that conformed most closely to a Kozak consensus motif (A/G)CCATGG was that at 420–422 (AGCATGG). Our in vitro transcription/translation results indicated that *STAG1/PMEPA1* protein synthesis could be initiated in vitro from the first or second ATG as well as from the third ATG. Use of the second or third ATG would result in truncation of the putative extracellular domain of *STAG1/PMEPA1* by four and 33 amino acids,

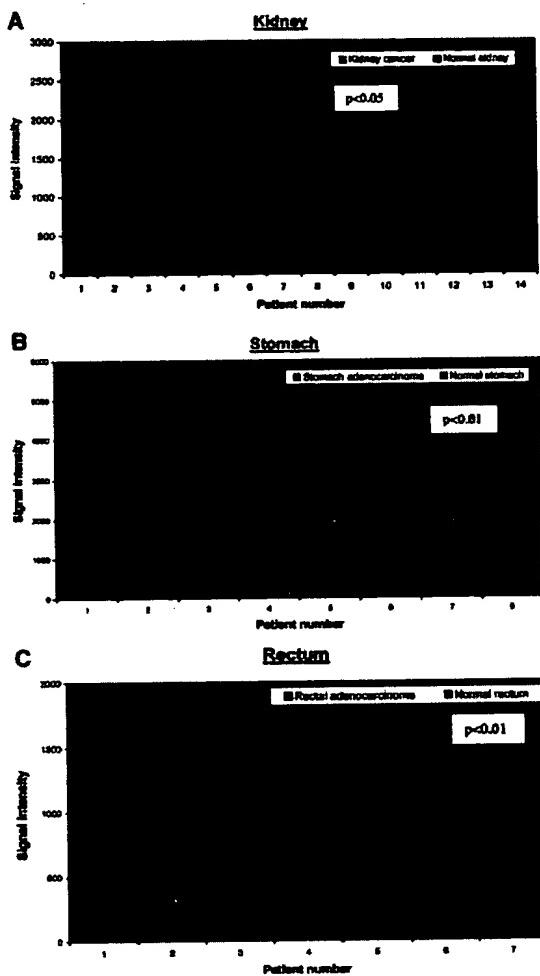
respectively. It will be important to determine which of these potential translation initiation sites is functional in vivo and the effect of protein truncation on *STAG1/PMEPA1* protein function. In addition, the molecular weights obtained in vitro for *STAG1/PMEPA1* of 36 kDa and 39 kDa were higher than the predicted molecular weights of 28 kDa and 32 kDa, respectively, indicating posttranslational modification of the protein. In support of this theory, there were a number of consensus sequences for posttranslational modifications, including glycosylation and N-myristylation sites.

Although the role of *STAG1/PMEPA1* is yet to be elucidated, the protein contained a putative transmembrane domain and motifs that suggest that it may bind SH3- and WW domain-containing proteins. SH3 domains are often present in eukaryotic signal transducing and cytoskeletal proteins and mediate such functions as protein-protein interactions and cell compartmentalization. These domains bind proline-rich sequences containing the core motif PXXP, where X denotes any amino acid [18]. Although the WW domain resembles the SH3 domain functionally by displaying affinity toward proline-rich ligands, their structures are distinct. WW domains, found in proteins that participate in cell signaling and regulation, form a binding pocket for ligands containing PPXY or PPLP core motifs, which usually are flanked by additional prolines [18]. The PPXY motif has been observed in a number of transcription factors, where it may play a role in transcriptional activation. Accordingly, *STAG1/PMEPA1* may function by aggregating signaling molecules localizing the protein complex on the inner surface of a cellular membrane. Our analysis indicated that *C18orf1* also contains potential SH3 and WW domain binding motifs.

Northern analysis and semiquantitative RT-PCR of RCC and normal kidney paired samples confirmed the upregulation of *STAG1/PMEPA1* in RCC. In addition, our analysis of the EST clones corresponding to *STAG1/PMEPA1* (Unigene EST clusters Hs.83883 and Hs.4299, containing 92 and 114

**Figure 3.** (A) Northern blot analysis of *STAG1/PMEPA1* expression in 16 normal human tissues. Hybridization was performed with cRNA probes generated from (i) the *STAG1/PMEPA1* 5' end (nucleotides 1–1403) and (ii) the *STAG1/PMEPA1* 3' UTR (nucleotides 4429–4763). (iii) A  $\beta$ -actin control probe was used to verify equivalent loading of RNA in each lane. (B) Graphical representation of signal intensities after hybridization with a probe generated from nucleotides 1–1403 of *STAG1/PMEPA1* cDNA to a Clontech Multiple Tissue Expression array containing polyA+ RNA from 76 different human tissues and cell lines. Tissues arrayed: 1, whole brain; 2, cerebral cortex; 3, frontal lobe; 4, parietal lobe; 5, occipital lobe; 6, temporal lobe; 7, cerebral cortex; 8, pons; 9, cerebellum, left; 10, cerebellum, right; 11, corpus callosum; 12, amygdala; 13, caudate nucleus; 14, hippocampus; 15, medulla oblongata; 16, putamen; 17, substantia nigra; 18, accumbens nucleus; 19, thalamus; 20, pituitary gland; 21, spinal cord; 22, heart; 23, aorta; 24, atrium, left; 25, atrium, right; 26, ventricle, left; 27, ventricle, right; 28, interventricular septum; 29, apex of heart; 30, esophagus; 31, stomach; 32, duodenum; 33,

jejunum; 34, ileum; 35, iloecum; 36, appendix; 37, colon, ascending; 38, colon, transverse; 39, colon, descending; 40, rectum; 41, kidney; 42, skeletal muscle; 43, spleen; 44, thymus; 45, peripheral blood leukocyte; 46, lymph node; 47, bone marrow; 48, trachea; 49, lung; 50, placenta; 51, bladder; 52, uterus; 53, prostate; 54, testis; 55, ovary; 56, liver; 57, pancreas; 58, adrenal gland; 59, thyroid gland; 60, salivary gland; 61, mammary gland; 62, leukemia, HL-60; 63, HeLa S3; 64, leukemia, K-562; 65, leukemia, MOLT-4; 66, Burkitt's lymphoma, Raji; 67, Burkitt's lymphoma, Daudi; 68, colorectal adenocarcinoma, SW480; 69, lung carcinoma, A549; 70, fetal brain; 71, fetal heart; 72, fetal kidney; 73, fetal liver; 74, fetal spleen; 75, fetal thymus; 76, fetal lung. (C) Semiquantitative RT-PCR for *STAG1/PMEPA1*. RT-PCR was performed on six female (1–6) and six male (7–12) paired RCC (R) and normal kidney (N) samples. Product sizes are indicated at the right.  $\beta$ -microglobulin was used as a control for cDNA synthesis, and no template PCR was performed as a negative control (–ve).



**Figure 4.** Graphical representation of signals obtained by probing a Matched Tumor/Normal Expression array containing cDNA from tumor and corresponding normal tissues from individual patients with a variety of cancers. Those tissues showing differential expression (kidney, RCC, stomach, and rectum—Figure 4A-C, respectively) are represented. The numbers on the y-axis denote arbitrary units obtained from densitometry analysis of the Matched Tumor/Normal array using ImageQuant software. Statistical analysis of cancer and normal samples for each tissue was performed using the Student's *t* test to obtain a *P* value.

clones, respectively), approximately 50% of the clones were found to be derived from tumor tissues. This is a significantly higher number than the general representation of ESTs from tumor tissue in EST database ( $P < 0.001$ ), which has been reported as only 12% [19]. ESTs found to match *STAG1/PMEPA1* were frequently from a pancreas adenocarcinoma library, followed by a stomach cancer library. Hybridization of *STAG1/PMEPA1* to a

tumor/normal matched array showed significantly increased expression in many stomach, rectal, and kidney cancers. Although only a small sample number has been examined and there are varying levels of expression between individual patients, these preliminary results indicate that the association of *STAG1/PMEPA1* with tumorigenesis warrants further investigation. Recently, comparative genomic hybridization was used to screen 47 primary stomach cancers for changes in the number of copies of DNA sequences [20]. High-level amplification was noted at a number of sites, including 20q13, and the most frequent alteration was the gain of 20q in 55% of cases.

In normal tissues, *STAG1/PMEPA1* was expressed abundantly only in the prostate. In a recent report *STAG1/PMEPA1* was identified from the prostate cancer cell line LNCaP using serial analysis of gene expression for genes induced in response to synthetic androgen R1881 treatment [10]. The expression of *STAG1/PMEPA1* in LNCaP cells was induced by androgen in a time-specific and dose-specific manner. *STAG1/PMEPA1* also was shown to be overexpressed in three of four androgen-independent secondary prostate tumors derived from a xenograft model. The authors did not note any increase of *STAG1/PMEPA1* expression in primary prostate tumors compared with normal prostate. Together with our findings that *STAG1/PMEPA1* was overexpressed in a number of solid tumors originating from tissues not highly regulated by androgens and that *STAG1/PMEPA1* was expressed in RCC tumors from both male and female patients, these findings suggest that this gene has other important tissue-specific regulatory mechanisms. The finding of alternate exon 1 sequences also suggests that tissue-specific regulatory sequences, which may play a role in this process, may be present in the promoter regions 5' of these alternate exons.

In summary, we have characterized the *STAG1/PMEPA1* gene, which encoded a putative transmembrane protein containing potential binding sites for proteins with SH3 and WW domains. *STAG1/PMEPA1* was highly expressed in normal prostate, and its expression also was upregulated in solid tumors of the kidney, stomach, and rectum. The functional role of this gene in the normal prostate and in tumorigenesis warrants further investigation.

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## REFERENCES

- Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* 1992;257(5072):967-971.
- Chen LC, Manjeshwar S, Lu Y, et al. The human homologue for the *Caenorhabditis elegans* *cul-4* gene is amplified and overexpressed in primary breast cancers. *Cancer Res* 1998; 58(16):3677-3683.
- Cole KA, Chuaqui RF, Katz K, et al. cDNA sequencing and analysis of *POV1* (PB39): A novel gene upregulated in prostate cancer. *Genomics* 1998;51(2):282-287.
- Mok SC, Chan WY, Wong KK, et al. *DOC-2*, a candidate tumor suppressor gene in human epithelial ovarian cancer. *Oncogene* 1998;16(18):2381-2387.
- Kocher O, Cheresh P, Brown LF, Lee SW. Identification of a novel gene, selectively upregulated in human carcinomas, using the differential display technique. *Clin Cancer Res* 1995;1(10):1209-1215.
- Ivanov SV, Kuzmin I, Wei MH, et al. Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. *Proc Natl Acad Sci U S A* 1998;95(21):12596-12601.
- Stassar MJ, Pitzer C, Zoller M. Downregulation of TNF receptor-associated protein-2/p97 in renal cell carcinoma. *Oncol Res* 1999;11(2):85-90.
- Thrash-Bingham CA, Tartof KD. aHIF: A natural antisense transcript overexpressed in human renal cancer and during hypoxia. *J Natl Cancer Inst* 1999;91(2):143-151.
- Rae FK, Stephenson S-A, Nicol DL, Clements JA. Novel association of a diverse range of genes with renal cell carcinoma as identified by differential display. *Int J Cancer* 2000;88:726-732.
- Xu LL, Shanmugam N, Segawa T, et al. A novel androgen-regulated gene, PMEPA1, located on chromosome 20q13 exhibits high level expression in prostate. *Genomics* 2000; 66:257-263.
- Kovacs G, Akhtar M, Beckwith B, et al. The Heidelberg classification of renal cell tumours. *J Pathol* 1997;183(2): 131-133.
- Guinan P, Sabin LH, Algaba F, et al. TNM staging of renal cell carcinoma: Workgroup No. 3. Union International Contre le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). *Cancer* 1997;80(5):992-993.
- Bentley S, Bassam BJ. A robust DNA amplification finger-print system applied to the analysis of genetic variation within *Fusarium oxysporum* f.sp. *cubense*. *J Phytopathol* 1996;144:207-213.
- Sheets M, Ogg S, Wickens M. Point mutations in AAUAAA and the poly(A) addition site: Effects on the accuracy and efficiency of cleavage and polyadenylation in vitro. *Nucleic Acids Res* 1990;18(19):5799-5805.
- Yoshikawa T, Sanders AR, Esterling LE, Detering-Wadleigh SD. Multiple transcriptional variants and RNA editing in *C18orf1*, a novel gene with LDLRA and transmembrane domains on 18p11.2. *Genomics* 1998;47: 246-257.
- Kyte J, Doolittle RF. A simple method for displaying the hydrophobic character of a protein. *J Mol Biol* 1982;157: 105-132.
- <http://www.expasy.ch>
- Kay B, Williamson M, Sudol M. The importance of being proline: The interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB J* 2000;14: 231-241.
- Schroer A, Schneider S, Ropers H, Nothwang H. Cloning and characterization of UXT, a novel gene in human Xp11, which is widely and abundantly expressed in tumor tissue. *Genomics* 1999;56(3):340-343.
- Nakanishi M, Sakakura C, Fujita Y, et al. Genomic alterations in primary gastric cancers analyzed by comparative genomic hybridization and clinicopathological factors. *Hepatogastroenterology* 2000;47(33):658-662.

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